27 Schwartz, W.J., and Gainer, H., Suprachiasmatic nucleus: use of ^{14}C-labelled deoxyglucose uptake as a functional marker. Science *197* (1977) 1089–1091.
28 Smith, G.P., and Epstein, A.N., Increased feeding in response to decreased glucose utilization in the rat and monkey. Am. J. Physiol. *217* (1969) 1083–1087.
29 Wicks, A.N., Drury, D.R., and Mortia, T.N., 2-Deoxyglucose, a metabolic block for glucose. Proc. Soc. exp. Med. *89* (1955) 579–582.
30 Wicks, A.N., Drury, D.R., Nakada, H.I., and Wolfe, J.B., Localization of the primary metabolic block produced by 2-deoxyglucose, J. biol. Chem. *224* (1957) 963–969.
31 Young, J.K., Nance, K.M., and Gorski, R.A., Effects of diet upon hypothalamic vulnerability of gold thioglucose in male mice. Brain Res. Bull. *4* (1979) 69–74.

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In vitro analysis of prothoracicotropic hormone specificity and prothoracic gland sensitivity in Lepidoptera[1]

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Summary. The kinetics of prothoracicotropic hormone (PTTH) activation of pupal prothoracic glands (PG) of the cabbage army worm, *Mamestra brassicae*, and silkworm, *Bombyx mori*, were determined in vitro. Activation was assessed by comparing the increase in the rate of ecdysone synthesis by one member of a PG pair incubated with a PTTH preparation from pupal brains with the basal rate of synthesis of the other PG incubated without PTTH. A time course of ecdysone synthesis revealed that *Bombyx* PTTH extract activated *Bombyx* PG, and *Mamestra* PTTH extract activated *Mamestra* PG. Dose responses of activation of *Bombyx* and *Mamestra* PG by their respective PTTH were saturable and were indicative of neurohormonal activation. The *Bombyx* PG were half-maximally activated (A_{50}) by far less PTTH than *Mamestra* PG, 0.34 and 0.91 brain equivalents, respectively. Heterologous dose response of activation studies, in which PTTH and PG from *Mamestra*, *Bombyx* and the tobacco hornworm, *Manduca sexta*, were assayed for interspecific PG sensitivity and PTTH specificity, revealed cross-reactivity among the three PTTH-PG axes, with *Manduca* PG being more sensitive to the PTTH of the other species and *Bombyx* PTTH being the most effective in activating the PG of the other two species.

The prothoracicotropic hormone (PTTH) is a peptide neurohormone[9,15] synthesized by specific neurosecretory cells in the insect brain[5,15] and released from its neurohemal organ, the corpus allatum[4] in *Manduca sexta*, into the hemolymph at specific times during insect development[9]. Once released, PTTH activates the prothoracic glands (PG) to synthesize and release ecdysone which is converted to 20-hydroxyecdysone by tissues other than the PG[16,19]. It is 20-hydroxyecdysone, or possibly a combination of ecdysone and 20-hydroxyecdysone, that elicits the molting process. Juvenile hormone acts in conjunction with the ecdysteroids as a modulator of cellular events in target tissues[16].

The ecdysteroids and juvenile hormones from a variety of insects have been characterized and these hormones appear to be ubiquitous, exhibiting only subtle molecular heterogeneity[16]. Although the chemical characterization of PTTH has been a matter of intensive study, the structure of this elusive peptide has not yet been elucidated[8,9,15]. Therefore, the only available information pertaining to the degree of structural conservation of PTTH among insect species comes from classical brain transplantation studies and in vivo bioassays, which suggest that this neurohormone may be far less conserved structurally than are the ecdysteroids and juvenile hormones[14].

With the development of a sensitive and specific in vitro assay for PTTH that quantifies activation of the PG by the neurohormone, it is now possible to investigate the cross-reactivity of PTTH among different insect species without purifying the PTTH of each species. This study was conducted to compare PTTH specificity, as well as PG sensitivity to PTTH, among *Bombyx mori*, *Mamestra brassicae* and *Manduca sexta*, representatives of 3 superfamilies of Lepidoptera; Bombycoidea, Noctuoidea and Sphingoidea.

Materials and methods

Animals. Larvae of the cabbage army worm, *Mamestra brassicae* (Noctuidae), and the tobacco hornworm, *Manduca sexta* (Sphingidae), were reared on artificial diets at 25 °C under a long-day photoperiod (LD 16:8)[6,20]. Larvae of the silkworm, *Bombyx mori* (Bombycidae), a F_1 hybrid strain resulting from a cross between strains J.124 and C.124, were reared on fresh mulberry leaves at 25 °C under constant light.

Gland selection and PTTH assay. Day-O pupal brains were selected as the source of PTTH since for *Manduca* this stage yielded a considerable quantity of PTTH[4,9]. The PG used in this study were also from day-O pupae, thus facilitating accurate stage selection for the comparative study. As described previously[11], brains were homogenized in Grace's medium, heat treated at 100 °C and centrifuged at 8000× g for 5 min. The supernatant, denoted PTTH extract, was not contaminated by ecdysteroids as shown by an ecdysteroid radioimmunoassay (RIA)[11]. The PTTH concentration of an extract was expressed in brain equivalents, i.e. fraction of a brain or number of brains in a given in vitro assay.

Prothoracic glands (PG) were from 0-6-h-old pupae, a time before the initiation of pharate-adult development in these lepidopterans. *Bombyx*, *Mamestra* and *Manduca* PG were dissected out in lepidopteran Ringers[11], rinsed for 10–30 min in Grace's medium, and then incubated individually in a 0.025 ml standing drop of Grace's medium in a well of a plastic multiwell tissue culture plate (Falcon 3008). One pupal PG of a pair was incubated with PTTH extract (experimental gland), while the contralateral PG was incubated in Grace's medium alone (control gland). PG were incubated for varying times depending upon experimental design, but always at 25 °C, under high humidity, and in the dark.

To quantify ecdysone synthesis by the PG in vitro, 0.01-ml aliquots of the 0.025-ml incubate were extracted with 0.3 ml methanol and the ecdysone content determined by RIA as described previously[5,11]. RIA activity is expressed as ng ecdysone synthesized by a PG or as an activation ratio (A_r), which is the quantity of ecdysone synthesized by an experimental PG (+PTTH) divided by that synthesized by the contralateral control PG (−PTTH)[11]. For the time course of synthesis studies, an aliquot of medium (0.01 ml) was removed from an incubate at selected times and an equal volume of comparable medium was immediately added back to maintain a constant incubation volume.

Expression of data. An assessment of PG sensitivity and PTTH specificity among the three species of Lepidoptera was made by comparing reciprocals of the ED_{50} values. The ED_{50} (effective dose) value is derived from a dose response curve of PG activation and represents the number of brain equivalents necessary to half-maximally activate a PG[11]. Alternatively, the reciprocal of the ED_{50} denotes the number of assays that can be conducted with one brain equivalent of PTTH extract, a value that can be used to quantify the PTTH content of a brain extract. By setting the highest value obtained (reciprocal of the ED_{50}) in the comparative studies (table) at 1.0, and converting all other values relative to it, a 'relative activity' value is obtained. An analysis of relative activities depends upon the assumption that the three species examined have chemically identical PTTH.

Results

Time course of ecdysone synthesis. The time course kinetics of ecdysone synthesis by day-O pupal PG of *Mamestra* (fig. 1) and *Bombyx* (fig. 2) were determined and compared to data obtained previously for *Manduca*[11]. PG incubated without PTTH extract (control glands) synthesized low (basal) levels of ecdysone during an 8-h incubation period totalling 0.3 and 4.0 ng ecdysone/gland for *Mamestra* and *Bombyx*, respectively, while *Manduca* PG under the same in vitro conditions synthesized 5.6 ng ecdysone/gland[11]. PG of each insect incubated with 1.0 brain equivalent of PTTH extract from the same species (experimental glands) exhibited increased rates of ecdysone synthesis which indicated that the PG were

Prothoracic gland (PG) sensitivity to homologous and heterologous extracts of prothoracicotropic hormone (PTTH)

Source of day-0 pupal PG	PTTH activity *M. sexta*		*M. brassicae*		*B. mori*	
	ED_{50}[a]	Relative activity[b]	ED_{50}[a]	Relative activity[b]	ED_{50}[a]	Relative activity[b]
M. sexta	0.16	1.0	0.35	0.46	0.17	0.94
M. brassicae	0.62	0.26	0.91	0.15	0.41	0.39
B. mori	0.94	0.17	1.21	0.13	0.34	0.47

PTTH was extracted from day-0 pupal brains. [a] The ED_{50} is derived from dose-response kinetics and is the number of brain equivalents necessary to elicit half maximal stimulation of the PG. The reciprocal of the ED_{50} denotes the number of assays that could be conducted with 1 brain equivalent and is, therefore, a relative measure of PTTH content. [b] The relative activity was obtained by arbitrarily setting the highest reciprocal of the ED_{50} at 1.0 (*M. sexta* PTTH on *M. sexta* PG) and converting all other reciprocal of ED_{50} values relative to that of *M. sexta*.

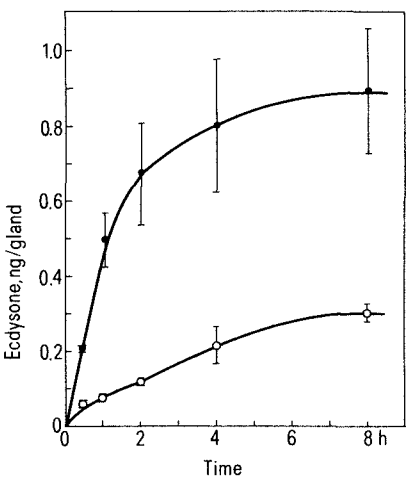

Figure 1. Time course of ecdysone synthesis by *Mamestra* prothoracic glands incubated in the absence of PTTH extract (○) and incubated in the presence of 1 brain equivalent of *Mamestra* PTTH extract(●). Each point represents the mean (SEM) of 4 separate determinations.

being activated. After 4 h of incubation, PTTH activated *Mamestra* and *Bombyx* PG synthesized 0.8 and 30 ng ecdysone/gland, respectively, compared to 22 ng/gland for *Manduca*. The activation ratios (A_r) observed for experimental vs control glands during this incubation time were ~6 for *Mamestra*, ~12 for *Bombyx* and ~4 for *Manduca* (fig. 3). The high A_r obtained for *Mamestra* and *Bombyx* PG in these time course studies established a kinetics basis for demonstrating saturable dose-dependent activation of the PG by PTTH extracts.

Dose response of PG activation by PTTH: Intraspecific. When PG from each species were incubated with the intraspecific PTTH extract, the glands were activated in a dose-dependent manner indicative of hormonal activation (fig. 3). Maximum activation (A_{max}) was obtained with 1.8 brain equivalents of PTTH for *Mamestra* (A_{max} ~13), 0.8 brain equivalent for *Bombyx* (A_{max} ~9) and 0.25 brain equivalents for *Manduca* (A_{max} ~4). The ED_{50} for each dose response of activation was 0.91, 0.34 and 0.16 brain equivalents of PTTH for *Mamestra*, *Bombyx* and *Manduca*, respectively (table). The ED_{50} values suggest that the *Manduca* PTTH-PG system is the most sensitive, followed by that of *Bombyx* and then *Mamestra*. This greater responsiveness of the *Manduca* system could be a result of the following: 1) the *Manduca* PG are more sensitive to PTTH; 2) the *Manduca* brain contains a greater amount of PTTH; 3) the *Manduca* PTTH is the most potent; and/or 4) the in vitro conditions are more favorable for the *Manduca* system.

Dose response of PG activation by PTTH: Interspecific. To obtain some insight into which of the above possibilities may account for the different in vitro behavior of the 3 PTTH-PG systems, interspecific studies were conducted in which dose responses of PG activation were generated using PG from one species and PTTH extracted from another. An ED_{50} analysis of the cross-reactivity between the PTTH extract of one species and the PG of another indicated that cross-reactivity occurred, but to varying degrees (table). PG of *Manduca* were activated equally by PTTH extracts from *Manduca* and *Bombyx*, while *Mamestra* PTTH extract was much less effective in activating these PG. In fact, the *Manduca* and *Bombyx* PTTH extracts were more effective in activating *Mamestra* PG than was *Mamestra* PTTH extract. Overall, *Bombyx* PTTH appeared to be the most potent. With *Bombyx* PG, *Bombyx* PTTH extract was most effective and the *Mamestra* PTTH extract the least effective.

In summary, these interspecific dose response studies conducted under conditions that do not account for possible variations in the molar concentration of PTTH in the different brains or the potential differences in sensitivity of the PG to PTTH, reveal that *Manduca* PG are more sensitive to PTTH extracts and that *Bombyx* PTTH extract is the most potent in terms of activating the PG.

Discussion

This study has established the efficacy of the in vitro PTTH assay developed for *Manduca*[11] in analyzing PTTH-PG relationships in other Lepidoptera. Indeed, the assay should be useful for insects representing other orders as well, since it has recently been utilized successfully with the brain-ring gland system of the fly, *Sarcophaga bullata*[18]. In addition, the data from the present study have also demonstrated varying degrees of cross-reactivity between the PTTH-PG systems of the 3 lepidopterans examined.

The hemolymph ecdysteroid titer of the newly pupated animals used in this study are remarkably similar, if not identical, i.e. ~300–350 ng/ml for *Bombyx*[13], *Manduca*[12] and *Mamestra*[3]. However, the volume of hemolymph in each of the insects is quite different; 0.18 ml, 0.6 ml and 1.4 ml for *Mamestra*[18], *Bombyx*[3]

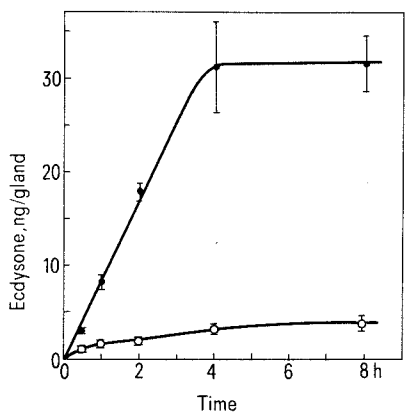

Figure 2. Time course of ecdysone synthesis by *Bombyx* prothoracic glands incubated in the absence of PTTH extract (○) and incubated in the presence of 1 brain equivalent of *Bombyx* PTTH extract (●). Each point represents the mean (SEM) of 4 separate determinations.

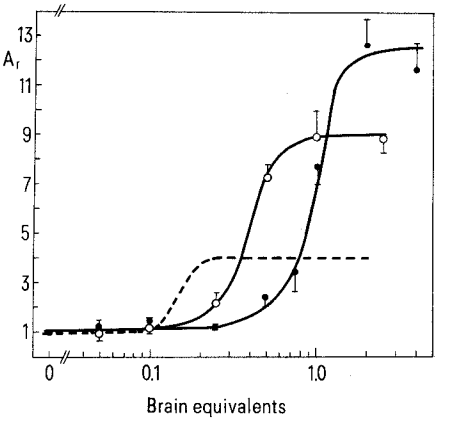

Figure 3. Dose responses of activation of prothoracic glands by *Bombyx* (○), *Mamestra* (●) and *Manduca* (---) intraspecific PTTH extracts. A_r denotes the activation ratio (see Materials and methods) and each point represents the mean (SEM) A_r from 3 separate assays.

and *Manduca*[7], respectively. For each species the hemolymph volume is proportional to the size of the animal: ~36% of the 0.5 g *Mamestra* pupa; ~30% of the 2.0 g *Bombyx* pupa; and ~35% of the 4 g *Manduca* pupa. As might be predicted, the data have shown that the in vitro rate of ecdysone synthesis by the PG of each species reflected this volume difference, i.e. the larger the hemolymph volume the greater the basal rate of ecdysone synthesis. It should also be noted that the *Mamestra* PG are the smallest of the 3 species studied.

Although the basal activity of the PG correlated with animal size (hemolymph volume), the in vitro kinetics data of gland activation by PTTH extract indicated that activated PG did not behave in a comparable manner. For example, the smaller and less active *Mamestra* PG required substantially greater amounts of *Mamestra* PTTH extract for activation than did the *Manduca* and *Bombyx* systems. In addition, once activated the A_{max} for *Mamestra* PG was comparable to that of *Bombyx* glands, values much greater than the A_{max} for *Manduca* PG. The reason(s) for these inconsistent kinetics data is not clear, but it is possible that the single incubation protocol employed to study PG activation for 3 different insects gave rise to these unexpected, apparent differences in the A_{max}. In this light, the kinetics data must be interpreted cautiously, particularly the A_{max} which can fluctuate dramatically for a given PTTH-PG system, as seen for *Bombyx*. Similar variations in A_{max} occur with the *Manduca* system; the values can vary from 3 to 8. The ED_{50} value, however, is reproducible irrespective of the changing A_{max}[10], thus making this a useful value for quantifying PTTH activity and for determining PG sensitivity.

Overall, the interspecific specificity and sensitivity analyses for *Manduca, Bombyx* and *Mamestra* PTTH and PG suggested the possibility of different gland sensitivities and PTTH specificities. Since the primary structure of PTTH is unknown, and no information is available regarding PTTH receptors in the PG, the interpretation of these data must be somewhat speculative. If it is assumed that the PTTH of the 3 species are chemically similar, or identical, then one must conclude that the *Manduca* PG are the most sensitive to PTTH stimulation and the *Mamestra* PG the least sensitive. Possible explanations for this apparent differential sensitivity of the PG include: 1) the size of endogenous ecdysone precursor pools; 2) the size of the glands; and 3) differences in PTTH receptors in the target glands, e.g. *Manduca* PG could possess more receptors and/or receptors having a greater affinity for PTTH. Conversely, the data could be due to considerable structural heterogeneity between the PTTH of the three species and nominal differences between the sensitivities of their PG.

Except for the case of *Manduca* PG sensitivity to *Bombyx* PTTH, there is no overlap in the order of PTTH specificity and PG sensitivity among the species investigated, suggesting that the neurohormones are similar in structure but not identical, and PG sensitivity to PTTH is species-specific. The actual quantity of PTTH in the brain of each pupa probably did not contribute significantly to the results of the interspecific studies since different PTTH extracts did not elicit comparable relative responses from the PG of the 3 species.

Irrespective of the degree and molecular basis of the cross-reactivity observed, the important finding from this study is that PTTH extract from one insect is capable of activating the PG of another. This observation indicates that the PTTH of these 3 lepidopterans are not species-specific, which in turn implies some degree of structural homology. The extent of this homology must await the purification and chemical characterization of the PTTH from each species. One PTTH present in the day-1 pupal brain of *Manduca* has now been purified[10] and its primary structure should be known in the near future. If antibodies are generated to this PTTH, one could probe the immunological similarities of this neurohormone among different insects without purifying and characterizing each PTTH.

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2 Agui, N., unpublished information.

3 Agui, N., and Hiruma, K., Ecdysteroid titer and its critical period during larval pupal ecdysis in the cabbage armyworm, *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Appl. Ent. Zool. *17* (1982) 144–146.

4 Agui, N., Bollenbacher, W.E., Granger, N.A., and Gilbert, L.I., Corpus allatum is release site for insect prothoracicotropic hormone. Nature *285* (1980) 669–670.

5 Agui, N., Granger, N.A., Gilbert, L.I., and Bollenbacher, W.E., Cellular localization of the insect prothoracicotropic hormone: in vitro assay of a single neurosecretory cell. Proc. natl Acad. Sci. USA *76* (1979) 5694–5698.

6 Agui, N., Ogura, N., and Okawara, M., Rearing of the cabbage armyworm, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and some lepidopterous larvae on artificial diet. Jap. J. appl. Ent. Zool. *19* (1975) 91–96.

7 Bassi, S.D., Bollenbacher, W.E., and Gilbert, L.I., unpublished information.

8 Bollenbacher, W.E., and Bowen, M.F., The Prothoracicotropic hormone, in: Endocrinology of Insects. Eds H. Laufer and R.G.H. Downer. A.R. Liss, New York 1983.

9 Bollenbacher, W.E., and Gilbert, L.I., Neuroendocrine control of postembryonic development in insects: the prothoracicotropic hormone, in: Neurosecretion, pp.361–370. Eds D.S. Farner and K. Lederis. Plenum Press, New York 1981.

10 Bollenbacher, W.E., and Gilbert, L.I., unpublished information.

11 Bollenbacher, W.E., Agui, N., Granger, N.A., and Gilbert, L.I., In vitro activation of insect prothoracic glands by the prothoracicotropic hormone. Proc. natl Acad. Sci. USA *76* (1979) 5148–5152.

12 Bollenbacher, W.E., Smith, S.L., Goodman, W., and Gilbert, L.I., Ecdysteroid titer during larval-pupal-adult development

of the tobacco hornworm, *Manduca sexta*. Gen. comp. Endocr. *44* (1981) 302–306.
13 Calvez, B., Hirn, M., and De Reggi, M., Ecdysone changes in the haemolymph of two silkworms *(Bombyx mori* and *Philosamia cynthia)* during larval and pupal development. FEBS Letters *71* (1976) 57–61.
14 Gilbert, L.I., and King, D.S., Physiology of growth and development: endocrine aspects, in: Physiology of Insecta, vol.I, pp.249-370. Ed. M. Rockstein. Academic Press, New York 1973.
15 Gilbert, L.I., Bollenbacher, W.E., Agui, N., Granger, N.A., Sedlak, B.J., Gibbs, D., and Buys, C.M., The prothoracicotropes: source of the prothoracicotropic hormone. Am. Zool. *21* (1981) 641–653.
16 Gilbert, L.I., Bollenbacher, W.E., Goodman, W., Smith, S.L., Agui, N., Granger, N.A., and Sedlak, B.J., Hormones controlling insect metamorphosis. Rec. Prog. Hormone Res. *36* (1980) 401–449.
17 Nagata, M., Seong, S., and Yoshitake, N., Variation of the haemolymph volume with larval development of the silkworm, *Bombyx mori*. J. seric. Sci. Tokyo *49* (1980) 453–454.
18 Roberts, B., Gilbert, L.I., and Bollenbacher, W.E., unpublished information.
19 Smith, S.L., Bollenbacher, W.E., and Gilbert, L.I., Studies on the biosynthesis of ecdysone and 20-hydroxyecdysone in the tobacco hornworm *Manduca sexta*, in: Progress in Ecdysone Research, pp.139–162. Ed. J.A. Hoffmann. Elsevier/North-Holland, Amsterdam 1980.
20 Vince, R., and Gilbert, L.I., Juvenile hormone esterase activity in precisely timed larvae and pharate pupae of *Manduca sexta*. Insect Biochem. *7* (1977) 115–120.

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Potential toxins of acute liver failure and their effects on blood-brain barrier permeability

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Summary. The effects of potential toxins of hepatic coma on the blood-brain barrier (BBB) permeability of the rat have been examined using the Oldendorf technique. Classical toxins of hepatic failure such as ammonia, methyl octanoate, mercaptans, and phenol caused significant increases in BBB permeability. A slight increase in permeability occcurred following infusion of peroxidized linoleic acid and unconjugated bilirubin but no increase after infusion of bile acids. *E. coli* endotoxin infused into rats following partial hepatectomy also increased the BBB permeability.

Livingstone et al.[13] in 1977 demonstrated an increase in BBB permeability in hepatectomized rats as they passed into a coma which was followed by cerebral oedema and death. No single toxin is likely to account for all the features of hepatic encephalopathy but raised plasma levels of water soluble toxins, such as ammonia and amino acids, as well as lipophilic toxins such as phenols[2], fatty acids[11], mercaptans, bile acids[1] and bilirubin have all been reported in liver failure. Recently there has been great interest in the disturbed balance of cerebral neurotransmitters secondary to changes in the brain and plasma amino acid profiles. Systemic endotoxemia may play a contributory role during liver failure since the function of Kupffer cells, which normally remove endotoxin, appears to be impaired in these patients[3]. The effects of toxins on BBB permeability have received little attention. In this paper we report changes in permeability of the BBB demonstrated using the Oldendorf technique[16] in normal rats following administration of a number of these toxins.

Method

Male Wistar rats weighing 250–300 g (King's College Hospital Medical School) were used for all studies. The rats were anesthetized with ether and i.p. sodium pentobarbitone (30 mg/kg) prior to administration of the toxins.

Administration of toxins. Phenol, endotoxin (*E. coli* 0111), unconjugated bilirubin, glycocholic, taurocholic, arachidonic and peroxidized linoleic acid were each infused into the femoral vein for 20 min prior to the measurement of the brain uptake index. Ammonium acetate and methyloctanoate were given as single i.p. doses. Ethanethiol was administered by inhalation to fully conscious rats in a fume chamber over a period of 5 min. The quantity of each toxin injected was based on the blood volume of the rat and was calculated to give a plasma concentration which would approximate to the pathologically raised levels seen in patients with fulminant hepatic failure. The bile acids were dissolved in 3 ml buffered albumin while the remaining toxins were dissolved in 3 ml of phosphate buffered saline prior to infusion by syringe pump over 20 min (Scientifica and Cook Electronics Ltd, London, UK). Infusion of phosphate buffered saline alone did not alter the brain uptake index in control rats. The estimated plasma concentrations of potential toxins, their routes of administration and the resultant brain uptake indices are shown in table 1.