Biochemical Pharmacology, Vol 26, pp. 223–228. Pergamon Press, 1977. Printed in Great Britain.

EFFECTS OF THE CARBOXYLESTERASE INHIBITOR BIS-(*p*-NITROPHENYL)-PHOSPHATE ON DISPOSITION AND METABOLISM OF HEXOBENDINE

NORBERT KOLASSA, ELISABETH TUISL and OTTO KRAUPP

Department of Pharmacology, University of Vienna, Waehringerstr. 13a, A-1090 Vienna, Austria

(*Received* 14 *April* 1976; *accepted* 31 *August* 1976)

Abstract—The effect of pretreatment with the carboxylesterase inhibitor bis-(*p*-nitrophenyl)-phosphate (BNPP) on the disposition and metabolism of intravenously-administered [^{14}C]hexobendine was studied in rats. Inhibition of hexobendine ester cleavage by BNPP produced a marked diminution in the concentrations of the hexobendine hydrolysis products, 3,4,5-trimethoxybenzoic acid and N,N'-dimethyl-N-[3-(3',4',5'-trimethoxybenzoxy)-propyl]-N'-3-hydroxypropyl-ethylendiamine in plasma and in the liver and kidneys. A simultaneous elevation occurred in the levels of demethylated metabolites following BNPP pretreatment. The concentration–time relationship of non-metabolized [^{14}C]hexobendine was not significantly altered by BNPP pretreatment. The results suggest that BNPP pretreatment produced a shift towards the dealkylating pathway in hexobendine metabolism. Consequently, esterase inhibition did not significantly affect the hexobendine plasma and tissue levels.

Studies on the disposition and metabolism of hexobendine, a potent coronary and cerebral vasodilatory substance [1–3], have shown that the cleavage of the two ester bonds is an essential process in hexobendine biotransformation in rats, leading to a rapid decline in plasma and tissue levels of the drug [4]. The question arises whether the metabolism and disposition of hexobendine can be influenced by administration of an esterase inhibitor in an attempt to increase the duration and intensity of the drug effects.

Among the inhibitors of carboxylesterases described in the literature [5, 6], BNPP* proved to be a relatively non-toxic, specific and irreversible inhibitor of liver and kidney carboxylesterases from different species [7]. Moreover, BNPP has been successfully used to inhibit amide cleavage of phenacetin and acetanilide *in vivo* with a consequent reduction in methaemoglobin formation by deacylated metabolites [8].

Since preliminary experiments showed a 90 per cent inhibition of hexobendine ester cleavage in a crude liver homogenate by 5×10^{-6} M BNPP (unpublished results), a study has been made of the effects of this esterase inhibitor on the metabolism and disposition of hexobendine *in vivo*.

MATERIALS AND METHODS

Materials. Male Wistar rats weighing 160–200 g were used in the experiments. They received a standard diet (T-79, 'Taco' Tagger & Co., Graz, Austria) and tap water *ad lib.* The rats were starved for about 18 hr before [^{14}C]hexobendine administration. Hexobendine-[carboxyl-^{14}C] with a sp. act. of 10 mCi/g and unlabelled hexobendine, *m*-monodemethylated hexobendine (ST 7255), partially hydrolyzed hexobendine with cleavage of one of the two ester bonds (ST 7221), the benzoic acid derivatives TMBA, 3-OH-DMBA and 4-OH-DMBA and all other reference substances of possible hexobendine metabolites were kindly provided by Chemie-Linz AG, Linz, Austria. BNPP was purchased from Merck AG, Darmstadt, Germany.

Drug administration and dosage. Pretreatment with BNPP (50 mg/kg) was given i.p. 18 hr and again 3 hr before hexobendine administration, and once more i.v. half an hour before drug administration. The BNPP solution, 5 mg/ml saline (0.9% w/v) was neutralized by addition of the appropriate amount of 0.15 N NaOH. The control rats received an equivalent volume of saline. [^{14}C]Hexobendine (0.5 mg/kg) was injected via the tail vein, the solution containing 0.1 mg/ml saline.

Measurement of ^{14}C disposition. As previously described [4], the rats were anaesthetized with ethylchloride either 3, 9, 27 or 81 min after [^{14}C]hexobendine administration. The abdomen and thorax were opened, the right ventricle was punctured and about 6 ml blood were withdrawn into a syringe filled with 0.1 ml heparin solution (40 mg/ml). The blood was immediately centrifuged (14000 *g*, 10 min, 2°); plasma and erythrocytes were separated and each fraction treated with 4 vol of methanol. The organs were excised and weighed in the following order: heart, lungs, liver, kidneys, spleen, skeletal muscle (M.quadriceps fem.) and brain. Homogenization (knife homogenizer, Bühler, Tübingen, Germany) was performed at high speed (40000 rpm for 30 sec) following the

* *Abbreviations used*—BNPP, bis-(*p*-nitrophenyl)-phosphate; ST 7255, *N,N'*-dimethyl-*N*-[3-(3'-hydroxy-4',5'-dimethoxybenzoxy)-propyl]-*N'*-[3-(3',4',5'-trimethoxybenzoxy)-propyl]-ethylendiamin; ST 7221, *N,N'*-dimethyl-*N*[3-(3',4',5'-trimethoxybenzoxy)-propyl]-*N'*-3-hydroxypropyl-ethylendiamin; TMBA, 3,4,5-trimethoxybenzoic acid; 3-OH-DMBA, 3-hydroxy-4,5-dimethoxybenzoic acid; 4-OH-DMBA, 4-hydroxy-3,5-dimethoxybenzoic acid.

addition of 20 ml methanol. The ^{14}C activity was measured in 1 ml supernatant together with 9 ml dioxane scintillator [9] in a Packard liquid scintillation spectrometer, model 3330. External standardization was undertaken to determine and correct for quench. The amount of methanol added together with the water content of the individual organs [10] was taken as extract volume for the calculation of the total ^{14}C activity in each organ.

Separation of metabolites. Depending on the ^{14}C-concentration of the individual samples 0.05–1.0 ml of the extracts were chromatographed on Silica gel 60-F-254 thin-layer plates (Merck, Darmstadt, Germany) together with unlabelled reference substances in two solvent systems: *n*-butanol/methyl ethyl ketone/acetone/NH_3(33%)/H_2O (40:40:25:8:13, by vol) was used for the determination of radioactivity in hexobendine (R_f = 0.92), ST 7255 (R_f = 0.80) and ST 7221 (R_f = 0.71). No measurable amount of ^{14}C was found in *p*-monodemethylated hexobendine (R_f = 0.47), *m,m'*-bisdemethylated hexobendine (R_f = 0.64) and *m*-demethylated ST 7221 (R_f = 0.55). TMBA (R_f = 0.36), 3-OH-DMBA (R_f = 0.23) and 4-OH-DMBA (R_f = 0.15) could not be separated from other demethylated and conjugated metabolites in this solvent. Hence, chloroform/ethyl acetate/*n*-hexane/formic acid (70:40:35:10, by vol) was used for the determination of radioactivity in TMBA (R_f = 0.75), 3-OH-DMBA (R_f = 0.57) and 4-OH-DMBA (R_f = 0.52). Since a clear-cut separation of the two OH-DMBAs was not achieved in each run, they were determined together and treated as one entity in this study. The amount of ^{14}C in 3,5-dihydroxy-4-methoxy-benzoic acid (R_f = 0.32) and 3,4-dihydroxy-5-methoxy-benzoic acid (R_f = 0.29) was below the detection level. In this second solvent all other above-mentioned hexobendine metabolites with at least one intact ester bond as well as any conjugates remained at the origin.

The individual spots on the thin-layer plates were visualized under u.v.-light and scraped into counting vials. Radioactivity was measured following the addition of 1 ml methanol/H_2O (2:1, by vol) together with 9 ml scintillation solution.

RESULTS

(1) *Total ^{14}C distribution.* The decay curves of total ^{14}C concentrations in the investigated organs of control and BNPP-pretreated rats following the i.v. administration of 0.5 mg/kg [^{14}C]hexobendine is shown in Fig. 1. The obtained pattern of ^{14}C distribution in the control rats is qualitatively and quantitatively comparable with previously-reported results [4]: the total ^{14}C activity in lungs, kidneys, spleen, liver, heart and skeletal muscle exceeded the plasma ^{14}C level; the erythrocytes and brain displayed a lower activity than the plasma. A considerable part of the ^{14}C measured in the brain extract was presumably due to ^{14}C in the extracellular space; hence, the true tissue content of ^{14}C in brain may be even smaller than the apparent values. The ^{14}C concentration in the spleen increased up to 27 min following [^{14}C]hexobendine administration, whilst in the other tissues the maximum ^{14}C values were recorded 3 or 9 min after drug administration and this was followed by a gradual decline. Pretreatment with 3 × 50 mg/kg BNPP did not produce any significant effect on ^{14}C levels in the different organs. However, the plasma ^{14}C concentrations were found to be markedly lower in BNPP-pretreated rats compared with control values (Fig. 1).

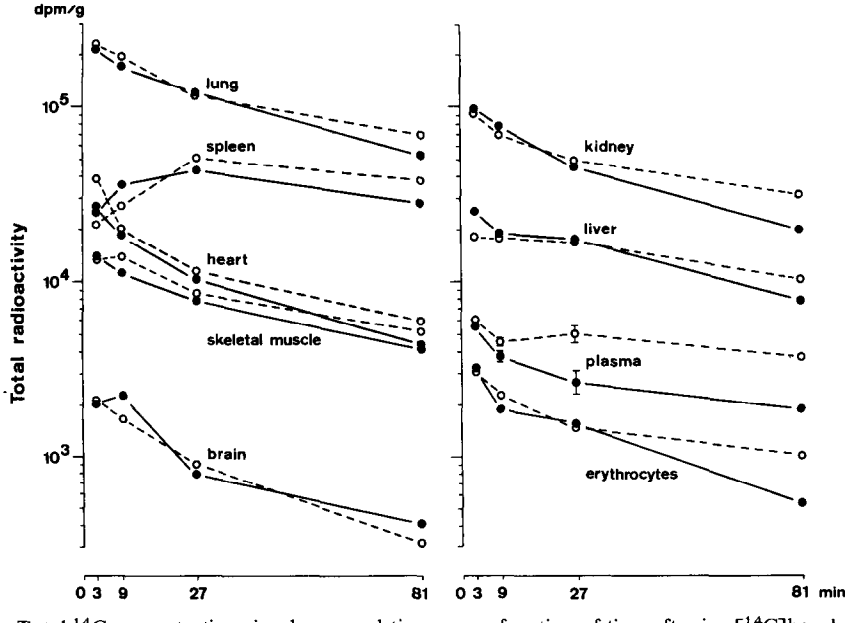

Fig. 1. Total ^{14}C concentrations in plasma and tissues as a function of time after i.v. [^{14}C]hexobendine administration in controls (○) and in BNPP-pretreated rats (●). Each point represents the mean value of three experiments. The standard error of the mean value is depicted in all cases in which a significant difference is obtained between mean values in controls and BNPP-pretreated rats at any particular time.

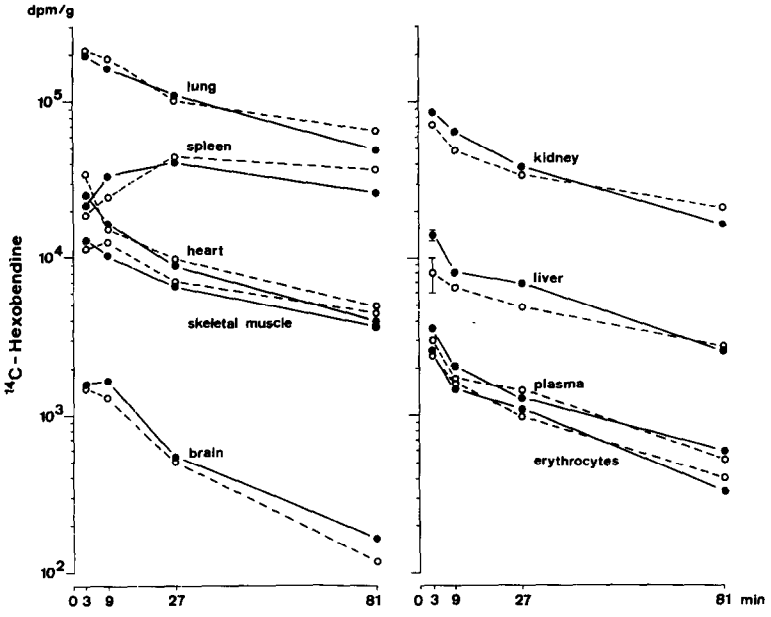

Fig. 2. [^{14}C]Hexobendine concentrations in plasma and tissues as a function of time after i.v. [^{14}C]hexobendine administration in controls (○) and in BNPP-pretreated rats (●). Each point represents the mean value of three experiments. The standard error of the mean value is depicted in all cases in which a significant difference is obtained between mean values in controls and BNPP-pretreated rats at any particular time.

(2)[^{14}C]*Hexobendine distribution.* Figure 2 shows the distribution of [^{14}C]hexobendine in the investigated organs. The [^{14}C]hexobendine concentration–time relationship was similar to that of total ^{14}C. Calculated tissue/plasma ratios were as follows: about 80 for the lungs, 30 for the kidneys, 8 for the heart, 5 for skeletal muscle, 4 for the liver, 0.8 for the erythrocytes and 0.4 for the brain throughout the observation period. The spleen was an exception in so far as the tissue/plasma ratio of [^{14}C]hexobendine rose from 6 at 3 min to 30 at 27 min after drug administration, in parallel with the prolonged accumulation already seen with total ^{14}C.

No major differences were observed between the tissue [^{14}C]hexobendine levels in control and BNPP-pretreated rats, except in the case of the liver, in which BNPP pretreatment led to somewhat higher [^{14}C]hexobendine levels 3 min following the administration of the labelled drug. Since the decay curves in Fig. 2 show no significant differences between the control and the BNPP-pretreated rats with regard to mean [^{14}C]hexobendine levels in any of the investigated tissues, it may be inferred that BNPP pretreatment did not significantly change hexobendine distribution and elimination during the observation period.

(3) *Distribution of ^{14}C-labelled metabolites.* Between 80 and 95 per cent of the total ^{14}C activity in control rats were identified as hexobendine in lungs, spleen, heart, and skeletal muscle and less than 5 per cent of total ^{14}C as TMBA. BNPP pretreatment induced only a small increase in the proportion attributable to hexobendine and a decrease in that due to TMBA in these organs. Other metabolites could not be identified with sufficient precision. Additional ^{14}C-labelled metabolites of [^{14}C]hexobendine were found in plasma, kidneys and liver; the appropriate activity curves are depicted in Figs. 3–5.

The significant difference in *plasma* total ^{14}C levels in control and BNPP-pretreated rats is mainly accounted for by the great difference in [^{14}C]TMBA concentrations, which were found to be reduced to

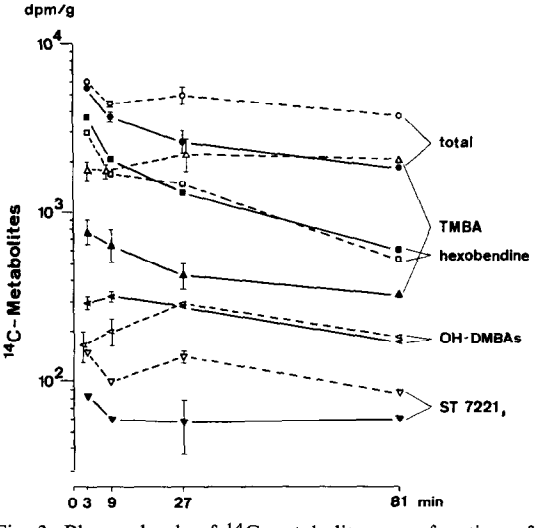

Fig. 3. Plasma levels of ^{14}C-metabolites as a function of time after i.v. [^{14}C]hexobendine administration in controls (blank symbols) and in BNPP-pretreated rats (solid symbols). Mean values of three experiments: total ^{14}C (○ ●), [^{14}C]hexobendine (□ ■), [^{14}C]TMBA (△ ▲), [^{14}C]ST7221 (▽ ▼) and [^{14}C]OH-DMBAs (◁ ◀). The standard error of the mean value is depicted in all cases in which a significant difference is obtained between mean values in controls and BNPP-pretreated rats at any particular time.

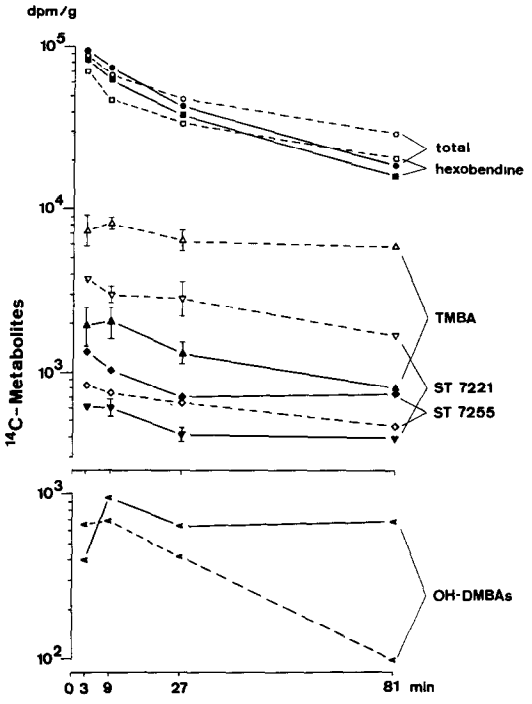

Fig. 4. Concentrations of ^{14}C-metabolites in the kidneys as a function of time after i.v. [^{14}C] hexobendine administration in controls (blank symbols) and in BNPP-pretreated rats (solid symbols). Mean values of three experiments: total ^{14}C (○ ●), [^{14}C]hexobendine (□ ■), [^{14}C]TMBA (△ ▲), [^{14}C]ST7221 (▽ ▼), [^{14}C]ST7255 (◇ ◆) and [^{14}C]OH-DMBAs (◁ ◀). The standard error of the mean value is depicted in all cases in which a significant difference is obtained between mean values in controls and BNPP-pretreated rats at any particular time.

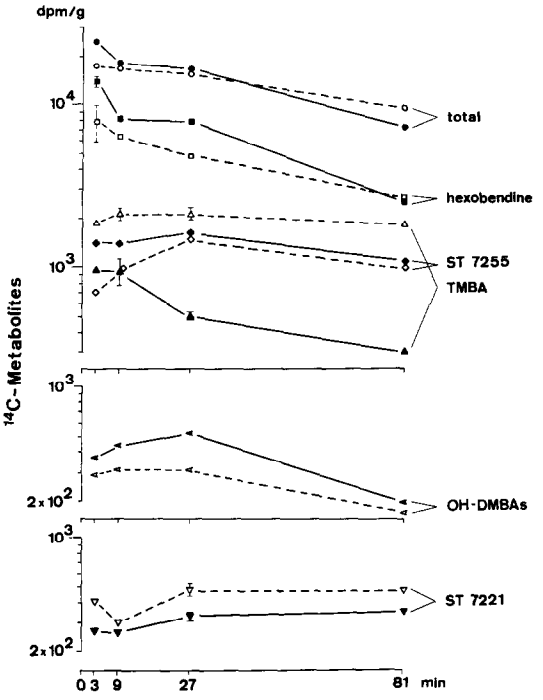

Fig. 5. Concentrations of ^{14}C-metabolites in the liver as a function of time after i.v. [^{14}C]hexobendine administration in controls (blank symbols) and in BNPP-pretreated rats (solid symbols). Mean values of three experiments: total ^{14}C (○ ●), [^{14}C]hexobendine (□ ■), [^{14}C]TMBA (△ ▲), [^{14}C]ST7221 (▽ ▼), [^{14}C]ST7255 (◇ ◆) and [^{14}C]OH-DMBAs (◁ ◀). The standard error of the mean value is depicted in all cases in which a significant difference is obtained between mean values in controls and in BNPP-pretreated rats at any particular time.

about one sixth of the control by BNPP pretreatment, whilst the levels of [^{14}C]hexobendine were not significantly influenced (Fig. 3). In control rats the plasma [^{14}C]TMBA level exceeded that of [^{14}C]hexobendine 27 min after drug administration; the relatively steady levels between 27 and 81 min indicate a state of equilibrium between the formation and elimination of TMBA during this period. By contrast, in BNPP-pretreated animals, the plasma [^{14}C]TMBA levels remained consistently far below those of [^{14}C]hexobendine and, moreover, the other hydrolysis product of hexobendine, namely ST 7221, was also significantly diminished. Three to six per cent of the total ^{14}C activity in the plasma of the control rats were identified as [^{14}C]OH-DMBAs originating from the demethylation of TMBA or, more probably, from the hydrolysis of a demethylated hexobendine metabolite (Fig. 6). In BNPP-pretreated rats the level of [^{14}C]OH-DMBAs was significantly elevated during the initial period following administration of hexobendine.

In the *kidneys* the main features, apart from the accumulation of [^{14}C]hexobendine, are the 3–4-fold higher [^{14}C]TMBA concentrations and the 20-fold higher [^{14}C]ST 7221 concentrations compared with the respective plasma values (Fig. 4). Whilst the combined [^{14}C]OH-DMBAs concentrations were found to be twice that in plasma, about one per cent of total ^{14}C in the kidneys was identified in the form of [^{14}C]ST 7255, a metabolite arising from demethylation of hexobendine (Fig. 6) which was not detectable in plasma by the method used. The influence of BNPP pretreatment presented a similar pattern to that in plasma: the levels of [^{14}C]TMBA and [^{14}C]ST 7221 were significantly depressed to about one fifth of the control values, whereas the levels of the demethylated metabolites, [^{14}C]ST 7255 and [^{14}C]OH-DMBAs, increased somewhat.

The concentration–time relationship of the different metabolites in the *liver* is given in Fig. 5. The [^{14}C]TMBA and combined [^{14}C]OH-DMBAs levels were of the same order as found in plasma. On the other hand, the concentrations of [^{14}C]ST 7221 were 3–6 times higher than in plasma and, in addition, [^{14}C]ST 7255 was present in a concentration of up to double that in the kidneys. Again, as in the kidneys, BNPP pretreatment produced a marked decrease in [^{14}C]TMBA levels in the liver, together with a slight fall in [^{14}C]ST 7221 and an increase in [^{14}C]ST 7255 and [^{14}C]OH-DMBAs.

DISCUSSION

This study of the distribution pattern of ^{14}C in the tissues following the i.v. administration of [^{14}C]hexobendine accords well with previously-reported results

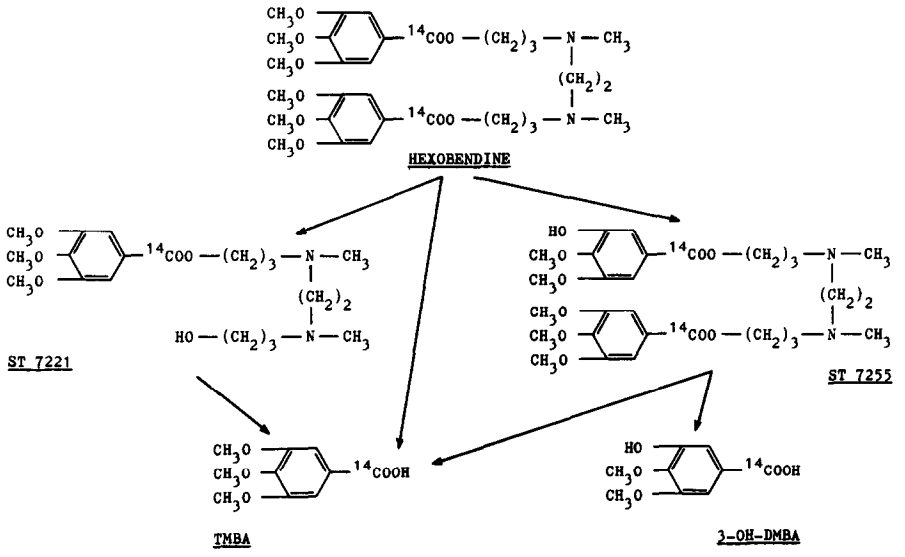

Fig. 6. Metabolic scheme of hexobendine.

[4]. The drug accumulated in the lungs, kidneys, spleen, heart, skeletal muscle and liver at concentrations exceeding the plasma level. [^{14}C]labelled metabolites were detected to a minor extent in the lungs, spleen, heart and skeletal muscle—organs with presumably low metabolic activity—but these metabolites contributed towards a high proportion of total ^{14}C-activity in the plasma, kidneys and liver.

The identified metabolites can be arranged in a metabolic scheme (Fig. 6) with two alternative pathways of hexobendine biotransformation: first, by cleavage of one ester bond of hexobendine, a partially hydrolyzed product of hexobendine (ST 7221) and TMBA are formed. Secondly, on dealkylation of hexobendine, demethylated products such as ST 7255, are formed, which are probably further metabolized by conjugation or ester cleavage yielding conjugates or OH-DMBAs.

The higher ST 7221 and ST 7255 concentrations in the kidneys and liver as compared with plasma levels point to the predominant role of these two organs in hexobendine ester cleavage and dealkylation. The accumulation of TMBA and OH-DMBAs in the kidneys is in agreement with the assumption that the benzoic acid derivatives formed may leave the organism by renal filtration and secretion as already shown in the case of TMBA [11]. On the other hand, the dealkylated hexobendine metabolites are probably excreted in the conjugated form by the liver analogous to the elimination pattern of dilazep [12], a drug which closely resembles hexobendine.

Pretreatment with BNPP, which was expected to retard hexobendine elimination by carboxylesterase inhibition, did not markedly change the disposition of total ^{14}C nor that of [^{14}C]hexobendine in the investigated organs, but significantly lowered the plasma ^{14}C level. A pronounced effect of BNPP was seen on the plasma and tissue levels of [^{14}C]TMBA and [^{14}C]ST 7221; these metabolites were reduced to one sixth of the respective control values as a consequence of esterase inhibition (Figs. 3–5). However, the goal of the present investigation to increase the hexobendine plasma and tissue levels over a prolonged period by esterase inhibition was not achieved. Consequently, other metabolic reactions, for instance dealkylation, should be taken into consideration as essential pathways in hexobendine metabolism.

Demethylated metabolites of hexobendine were demonstrated in the course of these experiments, *viz.* ST 7255 and the OH-DMBAs. The concentrations of these metabolites were markedly elevated by BNPP pretreatment. The increased formation of [^{14}C]OH-DMBAs may arise partly as a result of the higher level of ST 7255 and partly from an incomplete inhibition of ST 7255-splitting esterases by the participation of BNPP-insensitive species of carboxylesterases, as reported in the case of the cleavage of aliphatic amides [13]. Moreover, the poor penetrability of BNPP on account of its strong acidic properties (pK value = 2.48 [7]) may prevent the substance from reaching the intracellular sites, possibly involved in the formation and splitting of demethylated hexobendine metabolites.

It may be concluded that BNPP pretreatment produced a shift towards the dealkylating metabolic pathway in hexobendine biotransformation with the consequence that esterase inhibition did not markedly affect the hexobendine plasma and tissue levels.

Acknowledgements—We wish to thank Dr. L. Adler-Kastner for critically reviewing the manuscript. The gift of [^{14}C]hexobendine and reference substances by Chemie Linz AG is appreciated. The work was supported in part by the Fonds zur Förderung der wissenschaftlichen Forschung.

REFERENCES

1. O. Kraupp, P. Heistracher, E. Wolner and E. Tuisl, *Arzneimittel-Forsch.* **14**, 1086 (1964).
2. O. Kraupp, W. Grossmann, W. Stühlinger and G. Raberger, *Arzneimittel-Forsch.* **18**, 1067 (1968).
3. O. Kraupp, G. Nell, G. Raberger and W. Stühlinger, *Arzneimittel-Forsch.* **19**, 1691 (1969).
4. N. Kolassa, B. Dörr and K. Pfleger, *Arzneimittel-Forsch.* **25**, 1414 (1975).

5. W. N. Aldridge and E. Reiner, *Enzyme Inhibitors as Substrates*. North-Holland, Amsterdam (1972).
6. K. Krisch, in *The Enzymes*, (Ed. P. D. Boyer), Vol. V, 3rd edn. p. 43. Academic Press, New York (1971).
7. E. Heymann and K. Krisch, *Hoppe-Seyler's Z. physiol. Chem.* **348,** 609 (1967).
8. E. Heymann, K. Krisch, H. Büch and W. Buzello, *Biochem. Pharmac.* **18,** 801 (1969).
9. G. A. Bray, *Analyt. Biochem.* **1,** 279 (1960).
10. Documenta Geigy, Wissenschaftliche Tabellen, 6th edn., p. 486 (1960).
11. P. Numerof, M. Gordon and J. M. Kelly, *J. Pharmac. exp. Ther.* **115,** 427 (1955).
12. E. Schaumlöffel and M. Clausnitzer, *Arzneimittel-Forsch.* **22,** 1643 (1972).
13. F. R. Murphy, V. Krupa and G. S. Marks, *Biochem. Pharmac.* **24,** 883 (1975).