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CHROMATOGRAPHIC STUDY ON THE SPECIFICITY OF BIS-*p*-NITROPHENYLPHOSPHATE IN VIVO

IDENTIFICATION OF LABELLED PROTEINS OF RAT LIVER AFTER INTRAVENOUS INJECTION OF BIS-*p*-NITRO[^{14}C]PHENYLPHOSPHATE AS CARBOXYLESTERASES AND AMIDASES

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Summary

The procedure established to isolate the carboxylesterases E_1, E_2 and EA from rat liver (Arndt, R. and Krisch, K. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 589—598) was applied to characterize in vivo bis-*p*-nitro[^{14}C]-phenyl-*P*-labelled proteins. The peaks of radioactivity and of residual enzyme activities (hydrolysing methylbutyrate, *p*-nitrophenylacetate and acetanilide) were found in the same peaks after column chromatography and could be related to the well-defined esterases E_1, E_2 and EA. There is no indication of a nonspecific binding of bis-*p*-nitrophenyl-*P* or of one of its metabolites. The relative quantitative amounts of E_1, E_2 and EA were calculated to represent 40, 14 and 46%, respectively, of the total carboxylesterase content of rat liver. The relative amount of bound (not dialysable) radioactivity in rat liver depended on the survival time. During purification, the yield of enzyme activities corresponded to that of bound radioactivity, confirming the specificity of bis-*p*-nitrophenyl-*P* in vivo. Hence the radioactive metabolites of the inhibitor obviously do not possess binding affinities of quantitative importance to the rat liver proteins.

Introduction

Bis-*p*-nitrophenylphosphate has proved to be a rapid, irreversible and stoichiometric inhibitor of nonspecific carboxylesterases (EC 3.1.1.1) in vitro [1].

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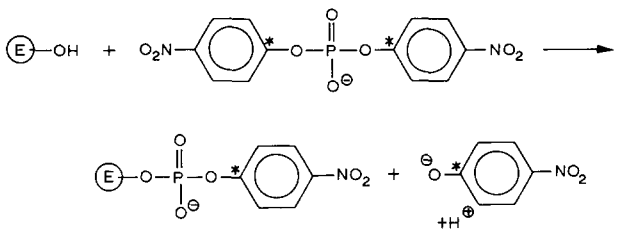

Fig. 1. Formula of the inhibitory reaction. E-OH represents a β-hydroxyl group of a serine residue at the active site of the enzyme. The position of the isotope is labelled by an asterisk.

Using bis-*p*-nitro[^{14}C]phenyl-*P*, carboxylesterases are labelled with a maximal binding capacity of half of the molar radioactivity of the inhibitor per mol of active sites.

The present study used a chromatographic approach to solve the question of the specific binding of bis-*p*-nitrophenyl-*P* for carboxylesterases in vivo. This question is of interest with regard to possible promising applications of bis-*p*-nitrophenyl-*P* in pharmacological and toxicological research and to clarify the role of the carboxylesterases in the metabolism of numerous compounds. In our experiments, we tested the specificity of bis-*p*-nitro[^{14}C]phenyl-*P* labelling in vivo on the well-defined system of rat liver carboxylesterases [2—6].

Some strong arguments for the specificity of bis-nitrophenyl-*P* in rat liver were already shown in previous investigations: After intravenous injections of bis-*p*-nitro[^{14}C]phenyl-*P*, we found a specific distribution pattern of radioactivity by whole body autoradiography [7]. This is in good agreement with esterase localization by histochemical methods. The subcellular distribution of radioactivity after differential centrifugation was identical to that of carboxylesterase activities [8]. The kinetics of the decrease of bound radioactivity parallels the reappearance of carboxylesterase activities [8,10]. The experiments here reported should verify on the molecular level, which of the carboxylesterases or other proteins of rat liver are responsible for the above-mentioned phenomena.

Materials and Methods

The animals used were female Wistar rats weighing 150 ± 2 g, kept on a standard diet ("Altromin R") and fasted for 24 h prior to killing.

Protein determination and enzyme assays was as reported previously [2] using as substrates acetanilide, methyl butyrate and *p*-nitrophenyl acetate.

The radioactivity assays were carried out as follows: 100—200 μl of the radioactive fluids were incubated in 2 vols. of Soluene-350 (Packard, U.S.A.) at 50°C. After adding of 15 ml of a scintillator mixture (4 g 2,5-diphenyloxazole plus 0.1 g 1,4-bis(5-phenyloxazole-2-yl) benzene made up to 1000 ml with toluene; scintillators were purchased from Packard, U.S.A.) and of 1—2 ml ethanol (analytical grade) the samples were measured in the Tricarb liquid scintillation counter (model 3380, Packard, U.S.A.).

Administration and dosage of bis-*p*-nitro[^{14}C]phenyl-*P*: Bis-4-nitro[1-^{14}C]-phenylphosphate (sodium salt) was purchased from Farbwerke Hoechst,

Germany. Its radioactive purity was tested by thin-layer chromatography and spectrophotometry. Unlabelled bis-*p*-nitrophenyl-*P* (Schuchhardt, Germany) was added to obtain the desired specific activity (1 μCi/mg). The dose of 0.031 g/kg body weight (4.65 mg bis-*p*-nitrophenyl-*P* in 1 ml 0.9% NaCl) were given by a single intravenous injection into the tail vein.

Preparation and purification procedure of rat liver carboxylesterases/amidases: At different times after application of the inhibitor (1, 2, 4, 8 and 24 h) the animals were decapitated and, immediately after bleeding ceased, the livers were removed. The livers containing the partially inhibited carboxylesterases (the extent of inhibition was found to be a function of survival time [8]) were homogenated and pooled. Solubilization, $(NH_4)_2SO_4$ precipitation, gel filtration and ion exchange chromatography were performed as described previously [2,3].

Radioactivity of the liver homogenates was measured before and after dialysis (3 days at 0—4°C against 5 mM phosphate buffer (pH 7.4), changing every 24 h). Subsequently the radioactivities of the dialysed homogenates and the dialysis buffers were determined and the relative amounts of dialysable radioactivity calculated. The recovery of radioactivity was almost 100%. The methyl butyrate-hydrolyzing activity did not change during dialysis, indicating that no reactivation took place.

Results

Solubilization and precipitation

The results of solubilization and $(NH_4)_2SO_4$ precipitation experiments are shown in Table I.

Percentage of bound radioactivity in rat liver after a single intravenous injection of bis-p-nitro[^{14}C]phenyl-P as a function of survival time

The yields of radioactivity after solubilization and $(NH_4)_2SO_4$ precipitation (67 and 22.8%, respectively) are considerably lower than the corresponding enzyme activities (Table I). Similar yields of labelled protein and of esterase activities would argue for the specificity of binding within the liver. To answer this question we determined the amount of bound radioactivity by dialysis in parallel experiments.

Fig. 2 shows the percentage of bound radioactivity as a function of the time from application of the labelled inhibitor up to the time of removal of organs (=survival time); the shorter the survival time, the higher the relative amounts of dialysable, non-bound radioactivity were found in the homogenates (at t = 30 min, only 30% of the total radioactivity are bound); complete binding of the radioactivity was observed not before 24 h after application of the inhibitor.

Homogenates of livers (with survival times of 24 h) were solubilized and precipitated by adding $(NH_4)_2SO_4$ in the same way as described for the pooled homogenates. These extracts contained a mean of 88% of the initial radioactivity and approx. 80% remained in the precipitate. Radioactivity could not be dialyzed from the extracts or the precipitates.

TABLE I

SOLUBILIZATION AND AMMONIUM SULFATE FRACTIONATION OF IN VIVO PARTIALLY INHIBITED CARBOXYLESTERASES AND AMIDASES FROM RAT LIVER AFTER INTRAVENOUS INJECTION OF BIS-*P*-NITRO[^{14}C]PHENYL-*P*

	Initial homogenate	Supernatant after solubilization	Sediment after $(NH_4)_2SO_4$ precipitation (40—70% saturation)
Volume (ml)	470	1 100	60.4
Protein			
Total (mg)	15 500	6 500	3 014
Yield (%)	100	41.9	19.5
Methyl butyrate-hydrolyzing activity			
Total (units)	2 120	1 820	1 477
Yield (%)	100	85.8	69.6
Acetanilide-hydrolyzing activity			
Total (units)	2.90	7.60	8.08
Yield (%)	100	90 *	90 *
p-Nitrophenyl acetate-hydrolyzing activity **			
Total (units)	2 820	2 453	2 010
Yield (%)	100	87	71.2
Radioactivity			
Total (dpm)	1940 000	1300 000	443 000
Yield of total radioactivity (%)	100	67	22.85
Yield of bound radioactivity *** (%)	100 ***	88 ***	80 ***

* Calculated values assuming an average increase of acetanilide activity solely due to solubilization (5) of about the threefold (= mean of 12 experiments); we found 'yields' of 262 and 278.6, respectively).

** Considering only the bis-*p*-nitrophenyl-*P*-sensitive fraction of the activity.

*** According to dialysis in parallel experiments.

Column chromatography

Gel filtration on Sephadex G-100 (Fig. 3): 2 active peaks (I and II) were found in the eluate using methyl butyrate, acetanilide and *p*-nitrophenyl acetate as substrates. The elution pattern of the enzyme activities correlated

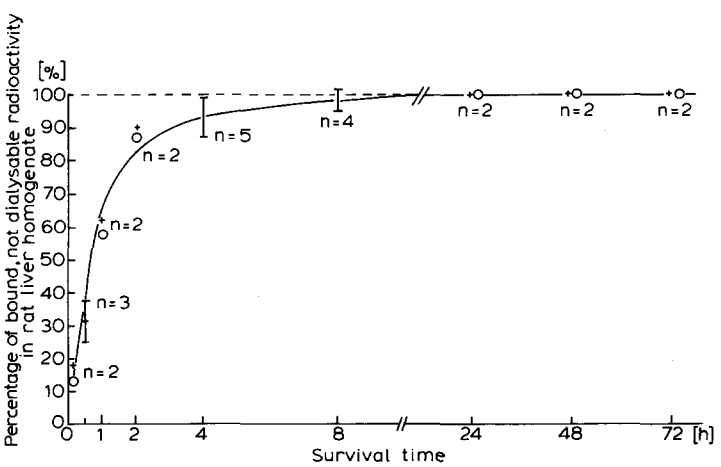

Fig. 2. Percentage of bound, not dialysable radioactivity of rat liver after a single intravenous injection of bis-*p*-nitro[^{14}C]phenyl-*P* (4.65 mg/kg body wt.) as a function of survival time. Each symbol represents one dialysis experiment; *n* = number of separately dialyzed homogenates.

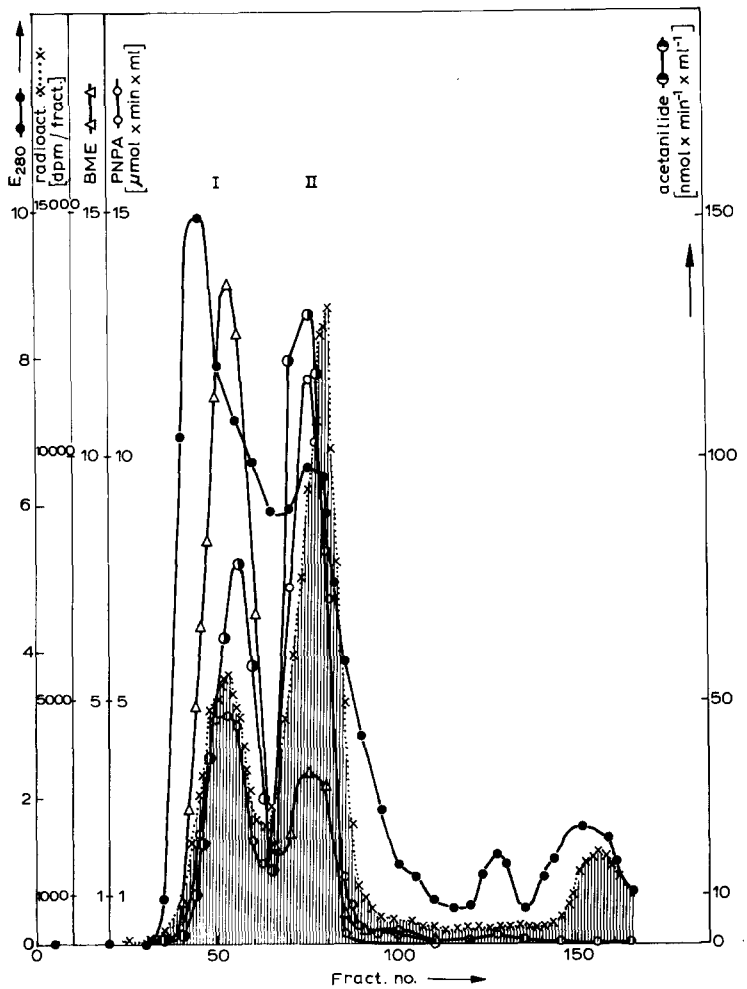

Fig. 3. Elution diagram after gel filtration on Sephadex G-100. 40.0 ml of the sediment after ammonium sulfate precipitation (saturation 40—70%) were applied to the column and eluated by $5 \cdot 10^{-3}$ M phosphate buffer, pH = 8.0. The gel column (2.5 · 90 cm) was equilibrated with the same phosphate buffer prior to chromatography. Fractions of 50 drops (approx. 3.25 ml) was sampled. Symbols: ●———●, extinction at 280 nm; △———△, methyl butyrate-hydrolyzing activity; ○———○, *p*-nitrophenyl acetate-hydrolyzing activity; ◑———◑, acetanilide-hydrolyzing activity; X· · · · · X, radioactivity.

closely to the radioactivity profile, although the second peak of high radioactive counts slightly deviated from the maximum of peak II (enzyme activity). The small radioactive peak, eluted at low molecular weight, is caused by *p*-nitrophenol and was identified by thin layer chromatography and subsequent radioscanning. The pooled fractions of peak I and peak II comprised 30.8% and 58.1%, respectively, of the radioactivity applied to the column (overall, a total of 101% recovery).

Chromatography on DEAE-cellulose (Fig. 4)

After gel filtration, fractions 63—90 (peak II) were pooled and a major part of the pool was applied to a DEAE-cellulose column. 3 radioactive peaks

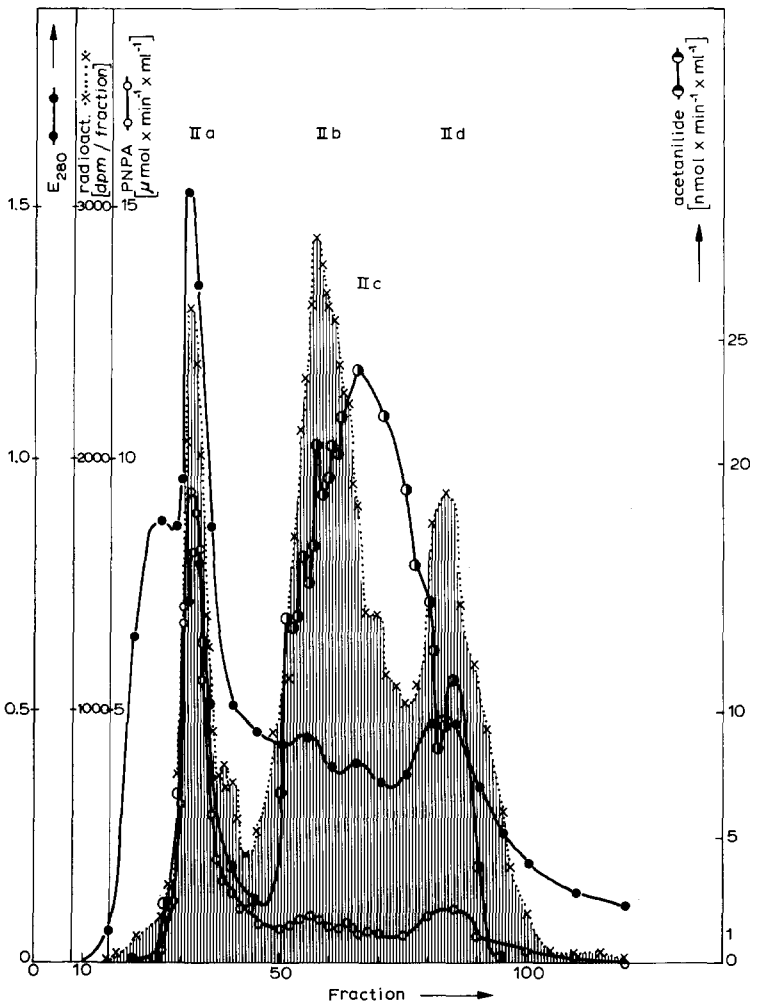

Fig. 4. Elution diagram after column chromatography on DEAE cellulose. 70.0 ml of pooled eluates (fractions 63—90) after gel filtration on Sephadex G-100 were applied to a DEAE-cellulose column (2.5 X 30.0 cm). The column was pre-washed by 200 ml $3 \cdot 10^{-5}$ M phosphate buffer, pH 8.0 and subsequently eluted using a phosphate buffer gradient of ascending molarity. The 4 vessels of the 4-chamber elution system each contained 150 ml of phosphate buffer at pH 8.0 in the following concentrations: 0.01, 0.04, 0.08 and 0.12 M. Fractions of 80 drops (= about 5.2 ml) were sampled. Symbols: see legend of Fig. 3.

appeared in the eluate. The position of the first radioactive maximum (peak II a), the main peak of *p*-nitrophenyl acetate-hydrolyzing activity and the first maximum of the acetanilide-hydrolyzing activity (fraction 32) all coincided. Smaller maxima of *p*-nitrophenyl acetate-hydrolyzing activity were situated in the region of peaks II b, c and d. Further, the radioactivity elution diagram shows two main maxima (II b and II d) which are paralleled by significant acetanilide and *p*-nitrophenyl acetate activities and the protein curve. The maximum of the acetanilide activity at peak II c coincides with a shoulder of the radioactivity curve.

Of the total radioactivity applied to the DEAE-cellulose column, peak II a

comprises 17.8%, peaks II b—d 76.1% (total recovery = 94.2%). The peaks of radioactivity and esterase activity presented in Figs. 1 and 2 may be easily and clearly related to the well-defined carboxylesterases E_1, E_2 and EA [2,3,5]: Peak I (Fig. 3) represents the esterase E_1 and the trimeric state of the enzyme EA which quantitatively is negligible [7]. Peak II (Fig. 3) represents the esterase E_1 and the monomer form of the enzyme E_2. Peak II a (Fig. 4) corresponds to the esterase E_2, peaks II b, c and d represent the heterogenous esterase EA. The methyl butyrate-hydrolyzing activities of EA and E_2 were expected to be very low, hence we did not determine this activity in the eluate of the DEAE-cellulose column.

Quantitative relations of the carboxylesterases E_1, E_2 and EA in rat liver

It is possible to calculate the quantitative relations of E_1, E_2 and EA in rat liver considering the different yields after extraction and $(NH_4)_2SO_4$ precipitation, because: (1) initially after application of the inhibitor, a total inhibition of the determined esterase and amidase activities was achieved [11]; (2) the bioligical halves-lives of the esterases E_1, E_2 and EA are approximately equal [9,10,12]. The amount of enzyme protein may be concluded from the bound radioactivity according to the following relationship: one mol of active centres of each of the 3 esterases is equivalent to 60000 g enzyme protein [3,5,6]. The proportion of bound radioactivities thus leads to the following quantitative relations: E_1 = 39.6%, E_2 = 13.7%, EA = 46.7%.

To see if these quantitative relations of the carboxylesterase isozymes of rat liver could be confirmed, we followed another, independent way. The average total carboxylesterase activities of a rat liver homogenate from an untreated 150 g female rat were 1144 ± 47.7 μmol methyl butyrate min^{-1} and 5980 ± 342 nmol acetanilide min^{-1}, respectively (n = 22 and 23, respectively; means ± standard deviation of means). These activities may, with a negligible deviation, be considered as caused by E_1 and EA, respectively [5].

Considering the known specific hydrolytic activities of the isolated enzymes E_1 and EA [4—6], the average content of a rat liver may be calculated as follows:

$$\frac{1140\ (\mu\text{mol methyl butyrate} \cdot \text{min}^{-1})}{500\ (\mu\text{mol methyl butyrate} \cdot \text{mg}^{-1} \cdot \text{min}^{-1})} = 2.28\ \text{mg}\ E_1$$

and

$$\frac{5.98\ (\mu\text{mol acetanilide} \cdot \text{min}^{-1})}{2.66\ (\mu\text{mol acetanilide} \cdot \text{mg}^{-1} \cdot \text{min}^{-1})} = 2.24\ \text{mg EA.}$$

An average 3-fold increase of the specific activity of the amidase EA upon solubilization [7] is taken into account (see footnote to Table I).

Since we know the total content of the bis-*p*-nitrophenyl-*P*-sensitive carboxylesterases of rat liver [8], the residual amount due to the E_2-isozymes may be calculated:

$$5.65 - 2.28 - 2.24 = 1.13\ \text{mg}\ E_2.$$

The quantitative relations of liver carboxylesterases ensuing from these values are: E_1 = 40.4%, E_2 = 20.0% and EA = 39.6%.

Discussion

The principle of labelling serine hydrolases by reaction with an irreversibly binding, radioactive inhibitor is frequently used, often to determine the number of active sites [11]. Ågren and Engström [12] ascertained the quantitative amount and the subcellular localization of serine hydrolases of rat liver by incubation of the subcellular fractions of a homogenate with diisopropylfluoro[^{32}P]phosphate and by subsequent isolation of [^{32}P]phosphorylserine. This group attempted to correlate the esterase activities (with *p*-nitrophenyl acetate as substrate) and the diisopropylfluoro-*P*-binding capacities of the soluble rat liver proteins within the eluates after ion-exchange chromatography [13]. Only a single peak of esterase activity was observed without diisopropylfluoro-*P*-binding capacity and no radioactive peak without esterase activity. Since these authors used in their in vitro experiments a nonspecific substrate and a nonspecific inhibitor (both reacting with numerous serine hydrolases), it was of interest to investigate the in vivo binding of an inhibitor that was more specific for carboxylesterases. We have made the first chromatographic separation of in vivo-labelled carboxylesterases. The radioactive peaks cochromatographed with the enzyme activities of the carboxylesterases E_1, E_2 and EA. The slight dissociation of the maxima of radioactivity and enzyme activity within peak II after gel filtration (Fig. 3) might be caused by changes of protein conformation due to the binding of ligands in this case resulting in a reduced molar volume. The splitting of the radioactivity curve, enzyme activity curve and protein curve into peaks II b, II c and II d after ion exchange chromatography (Fig. 4) results from the known heterogenity of charge of the esterase EA [5]. Whereas up to now bis-*p*-nitrophenyl-*P* was simply supposed to be a relative specific inhibitor according to some in vitro incubation experiments [1] these in vivo studies clearly show the specificity of bis-*p*-nitrophenyl-*P* for the rat liver carboxylesterases. This is in good agreement with other investigations we reported recently [7,8,10].

Since the yields of the protein bound radioactivity during purification procedure exactly correspond to the proportionately averaged amount of the yields of the 3 determined enzyme activities as could be ascertained by dialysis in parallel experiments, the proof of specificity presented here does not only concern the 19.5% of the total protein which had been submitted to chromatographic separation, but refers to the whole initial homogenate.

Heymann claimed in his report on the radioactivity-binding capacity of pig liver microsomes after incubation with bis-*p*-nitro[^{14}C]phenyl-*P* [14], that if carboxylesterases were the only inhibitor-binding microsomal proteins, one could calculate the esterase content from their specific radioactivity. However, one has to consider a possible protein binding of radioactive metabolites of bis-*p*-nitrophenyl-*P* as well. The main metabolites, *p*-nitrophenol and *p*-nitrophenylphosphate, are formed during incubation of bis-*p*-nitrophenyl-*P* with microsomes or homogenate, too; in vivo conjugates of *p*-nitrophenol are of additional importance [9]. *p*-Nitrophenylphosphate is produced enzymatically by phosphodiesterase IV which is present in rat liver and hydrolyzes bis-*p*-nitrophenyl-*P* as a substrate [15]. Such specific or unspecific binding of radioactive metabolites would result, however, in different rates of yield for bound

radioactivity and enzyme activities and hence may be excluded for rat liver.

The initially high amount of dialysable, non-protein-bound radioactivity in the liver (see Fig. 2) is partly due to an excess of unmetabolized inhibitor (the inhibition process takes only a few minutes for quantitative reaction [5]) and partly to the radioactive metabolites. The fact that not before 24 h after injection of bis-*p*-nitrophenyl-*P* all the liver radioactivity was bound, was confirmed by the time course of the decrease of total radioactivity in the liver which only follows first order kinetics after $t = 24$ h [8,10]. This is not incompatible with the results of Jandorf and Namara [17] who found that, after 4 h, all liver radioactivity is protein-bound: this was probably due to the use of only 0.5 mg diisopropylfluoro[^{32}P]phosphate/kg body weight which corresponds to about a sixtieth of our dose.

We found similar values for the relative occurrence of the carboxylesterase isozymes E_1, E_2 and EA in rat liver using two independent methods. Whether regarding the total enzyme activities of livers of untreated rats and considering the known specific activities of the isolated isozymes E_1 and EA or evaluating the relative size of radioactive peaks of the elution diagrams after chromatographic separation of bis-*p*-nitro[1-^{14}C]phenyl-*P*-treated material, the quantitative relation of the methyl butyrate- to the acetanilide-hydrolyzing enzyme was found to be approx. 1 : 1. So far, no information on these data was available in literature.

The specificity of bis-*p*-nitrophenyl-*P* in vivo which could be demonstrated by us for the rat liver suggests further animal experiments using bis-*p*-nitrophenyl-*P* to solve the question whether carboxylesterases are involved in the metabolism of a compound or not. But, moreover, we are encouraged to apply tritiated bis-*p*-nitrophenyl-*P* in an approach to the ultrastructural localization of the carboxylesterases, a problem which is in debate [17].

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