Journal of Chromatography, 256 (1983) 71–79
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,361

USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO STUDY THE CAERULOPLASMIN-CATALYSED OXIDATION OF BIOGENIC AMINES

I. SINGLE SUBSTRATE SYSTEMS

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(Received August 31st, 1982)

SUMMARY

Reversed-phase ion-pair chromatography was used to monitor the oxidation of four biogenic amines (adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine) by the copper-containing protein, caeruloplasmin. The methods are reproducible and sufficiently rapid to permit the handling of plasma sample batches. Kinetic parameters obtained using the method are in good agreement with those obtained by more traditional means of enzyme assay. Finally, the results support the view that more than one site on the enzyme, whether binding or oxidative, may be involved in the oxidation of biogenic amines, and the possible implications of this are briefly discussed.

INTRODUCTION

Caeruloplasmin, a protein from the α_2-globulin fraction of human plasma, has attracted considerable interest as it appears to perform several physiological functions. Its name derives from its role as the major copper transport protein, binding more than 95% of the circulating copper in human plasma[1]. It also possesses ferroxidase activity, thus mobilizing iron from storage sites to the plasma[2]. These two functions have been extensively studied and proved to be important physiological roles.

Caeruloplasmin has also been shown to catalyse the oxidation of certain biogenic amines, notably the catecholamines adrenaline, noradrenaline and dopamine and the indoleamine 5-hydroxytryptamine (5HT)[2]. Barrass and Coult[3] demonstrated that some psychotropic substances, such as lysergic acid N,N-diethylamide (LSD), can affect the relative rates of oxidation of these amines by caeruloplasmin and suggested that this enzyme, or a similar protein, could be responsible for regulating biogenic amine levels within the brain. Subsequently, the same workers isolated such a protein from brain tissue[4].

It is widely believed that many psychiatric conditions are due to an imbalance

0021-9673/83/0000–0000/$03.00

of neurotransmitter metabolism. Thus, it is conceivable that caeruloplasmin may play a role in the control of mental function.

As the brain enzyme, in man, is inaccessible for clinical studies, attention has been directed towards plasma caeruloplasmin, especially as a mechanism has recently been proposed whereby the plasma enzyme is transported to the cerebrospinal fluid via the choroid plexus[5].

Many spectrophotometric methods exist for determining the oxidase activity of caeruloplasmin using non-physiological substrates that produce highly chromogenic products[6]. However, the oxidation products of the biogenic amines show lower chromogenicity and, although these compounds have a strong UV absorbance, their absorption spectra overlap those of their native amines, making direct spectrophotometric assays unsuitable. Other methods used to monitor biogenic amine oxidation by caeruloplasmin include polarographic measurement of oxygen uptake, polarographic measurement of oxidized nicotinamide adenine dinucleotide (NAD^+) formation, or by spectrophotometrically following the rate of disappearance of reduced nicotinamide adenine dinucleotide (NADH)[3]. These methods are relatively insensitive, and may be non-specific in the presence of crude biological material such as serum, owing to other oxidative processes.

In recent years there has been increasing use of high-performance liquid chromatography (HPLC) to study enzyme reactions[7,8] as it offers several advantages over other methods of measurement. The specificity of the chromatographic separation eliminates the need for time-consuming extraction procedures and the sensitivity of modern HPLC detection systems allows the measurement of very low levels of enzyme activity. It may also be possible to detect products of any secondary reactions that may occur.

This paper describes the development of an HPLC method for studying biogenic amine oxidation by caeruloplasmin, either in purified form or in plasma. Following incubation of the enzyme with substrate, the amines and their oxidation products were rapidly separated using reversed-phase ion-pair chromatography and measured by UV detection.

EXPERIMENTAL

Materials and equipment

Adrenaline, noradrenaline, dopamine, 5HT, adrenochrome and human caeruloplasmin were obtained from Sigma (Poole, Great Britain). Stock solutions of the catecholamines ($1.4 \cdot 10^{-4}$ *M*) and 5HT ($3.5 \cdot 10^{-4}$ *M*) were prepared in 10^{-2} *M* hydrochloric acid and were stable for two weeks at 4°C. Dilutions of human caeruloplasmin were prepared in 0.25 *M* sodium chloride solution immediately before use. Methanol and sodium heptane sulphonate were supplied by Fisons (Loughborough, Great Britain). All other chemicals were purchased from BDH (Poole, Great Britain) and were of analytical-reagent grade. Centriflo CF 25 ultrafiltration cones were purchased from Amicon (Woking, Great Britain).

The chromatographic system consisted of an LC-XPD twin reciprocating pump (Pye Unicam, Cambridge, Great Britain); a Whatman LIB injection port (Whatman Labsales, Maidstone, Great Britain); a 100 × 4.6 mm I.D. column packed with ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain), particle

size 5 μm; a CE-212 variable-wavelength UV monitor (Cecil Instruments, Cambridge, Great Britain) and a Vitatron UR 401 recorder (Fisons, Loughborough, Great Britain). Spectrophotometric studies were carried out using a Pye Unicam SP 1800 instrument.

Blood was collected by venepuncture and allowed to coagulate. The serum was separated and stored at −20°C prior to analysis. Heparinized plasma was also found to be suitable for the assay.

The caeruloplasmin concentration of all sera, controls and purified enzyme standards were determined by radial immunodiffusion on M-Partigen plates (Hoechst Pharmaceuticals, Hounslow, Great Britain).

Adrenaline oxidation

An incubation tube, of final volume 1.2 ml, containing $1.75 \cdot 10^{-3}$ *M* adrenaline, $8 \cdot 10^{-2}$ *M* sodium acetate buffer (pH 5.5) and 50 μl of enzyme solution, was set up. Following incubation at 37°C for 45 min, the reaction was terminated by the addition of 50 μl of $1.5 \cdot 10^{-2}$ *M* sodium azide solution, and the tube stored on ice to await chromatography.

Duplicate 10-μl aliquots were injected into the chromatographic system. The mobile phase was $5 \cdot 10^{-2}$ *M* potassium phosphate buffer (pH 5.5), containing 10% (v/v) methanol and $2 \cdot 10^{-3}$ *M* sodium heptane sulphonate, at a flow-rate of 1.5 ml/min. The detection wavelength was 300 nm.

Noradrenaline oxidation

The procedure was as for adrenaline oxidation except that the incubation mixture contained $1.75 \cdot 10^{-3}$ *M* noradrenaline instead of adrenaline, and the methanol content of the mobile phase was reduced to 7.5% (v/v).

Dopamine oxidation

The procedure was similar to that used for adrenaline oxidation except that $1.75 \cdot 10^{-3}$ *M* dopamine replaced adrenaline in the incubation mixture and the methanol content of the mobile phase was increased to 17.5% (v/v), at a flow-rate of 1.3 ml/min.

5-Hydroxytryptamine oxidation

The incubation mixture contained $4.4 \cdot 10^{-3}$ *M* 5HT in place of adrenaline. The mobile phase contained $5 \cdot 10^{-2}$ *M* potassium phosphate buffer (pH 5.5), 22.5% (v/v) methanol and $9 \cdot 10^{-4}$ *M* sodium heptane sulphonate; the flow-rate was 1.0 ml/min. The detection wavelength was 315 nm.

Oxidation by serum enzyme

The incubation tubes were set up as described, with 50 μl of serum replacing the purified enzyme. Prior to chromatography, protein was removed by centrifuging through Amicon CF 25 cones for 5 min at 2000 rpm, and the protein-free filtrates were stored on ice. Because endogenous substances in the serum may produce peaks that interfere with the quantitation of the oxidation products, a blank was prepared for each serum, in which 10^{-2} *M* hydrochloric acid replaced the amine.

Standardization

For the three catecholamines, 5 μl of stock solution were added to 2.9 ml of 0.1 M acetate buffer (pH 5.5). Following addition of 0.1 ml of $3 \cdot 10^{-2}$ M potassium hexacyanoferrate(III) solution, the mixture was allowed to stand for 1 h at room temperature, in order to oxidize the amine completely to the corresponding aminochrome. Injection volumes of 10 μl of these mixtures contained 0.233 nmol of aminochrome. These standard solutions were stable for several hours on ice. Standardization of 5HT oxidation was based on the rate of product formation by known concentrations of caeruloplasmin.

For the catecholamines, it was found to be unnecessary to include an internal standard to compensate for variations due to syringe injection. The monitoring wavelength, 300 nm, is optimal for the aminochromes, but also permits the native amines to be detected at much lower sensitivity than at their optimum. As the amine is present in great excess, the two peaks of amine and aminochrome are of the same order of magnitude on the final chromatogram. It can be shown that even for sera with high oxidase activity, less than 1 % of the substrate is oxidized during the 45-min incubation period. Therefore, on the basis that the height of the amine peak is not significantly altered by the oxidation, this peak was used as an internal standard.

The absorption spectra for 5HT and its oxidation product did not permit a similar internal standardization procedure. As an exogenous internal standard may effect the oxidation of the amine, it was decided to correct for variations in injection volume by averaging triplicate injections of 5HT incubation mixtures.

Calculation of amine oxidation rate

Quantitation was based on measurement of peak height.

$$\text{Aminochrome injected } (A') = \frac{\text{Test aminochrome peak height}}{\text{Standard aminochrome peak height}} \cdot 0.233 \text{ nmol}$$

Amount of aminochrome produced by a ml of enzyme solution/serum per minute at 37°C

$$= A' \cdot \frac{1250}{10} \cdot \frac{1000}{50} \cdot \frac{1}{45}$$

$$= A' \cdot 55.6 \text{ nmol min}^{-1} \text{ ml}^{-1}$$

where

1250 = final volume of incubation mixture (μl);
10 = injection volume (μl);
50 = volume of enzyme solution/serum (μl);
45 = time of incubation (min).

Quality control

Each batch included a standard solution of purified caeruloplasmin prepared

by diluting 10 μl of stock enzyme with 1.99 ml of 0.25 *M* sodium chloride solution. Two separate pools of sera were aliquoted and stored at −20°C. These were also included in each batch.

RESULTS

The absorption spectra of adrenaline and adrenochrome solutions in 0.1 *M* acetate buffer (pH 5.5) are shown in Fig. 1. In order to determine the absorption characteristics of the amine oxidation products, difference spectra were obtained for each amine by spectrophotometrically scanning a buffered substrate solution following incubation with enzyme against the same solution from which the enzyme had been excluded. Adrenaline, noradrenaline and dopamine gave absorption spectra identical with that of adrenochrome, confirming the structural similarity of their oxidation products. The difference spectra obtained from the oxidation of 5HT is shown in Fig. 2.

The chromatographic conditions for each amine were chosen so that the amine and its oxidation product could be completely resolved in as short an analysis time as possible, thus permitting large batches of sera to be analysed within the time limit of aminochrome stability, which was at least 6 h when stored on ice. The separation of adrenochrome from adrenaline is shown in Fig. 3.

In order to select optimum incubation conditions for the assay, the rates of

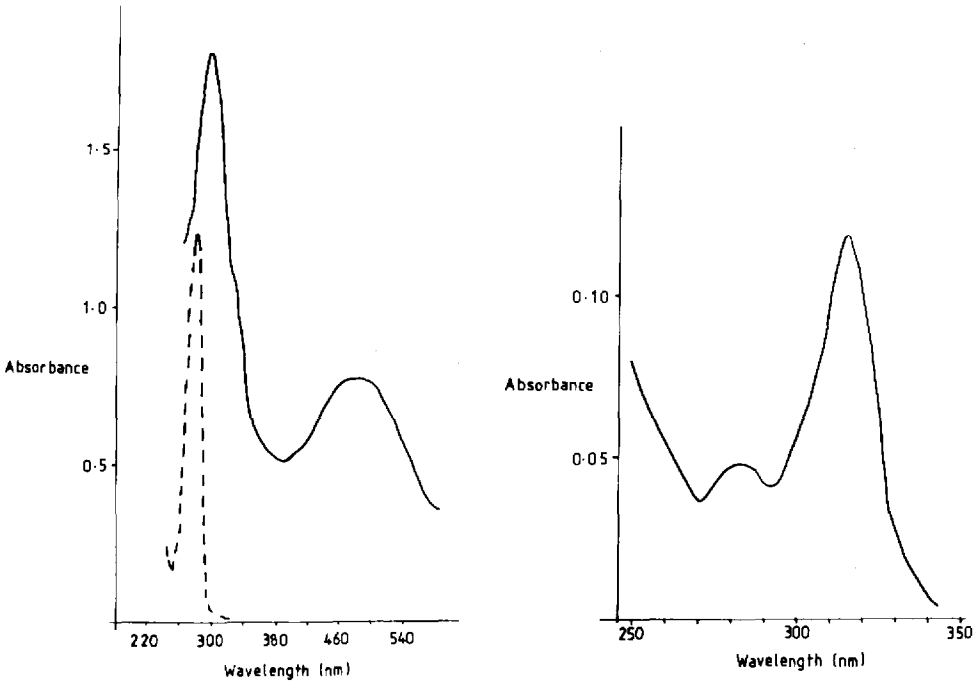

Fig. 1. Absorption spectra of $5 \cdot 10^{-5}$ *M* adrenaline (broken line) and $2 \cdot 10^{-4}$ *M* adrenochrome (solid line).
Fig. 2. Absorption spectra of the product of 5-hydroxytryptamine oxidation by caeruloplasmin.

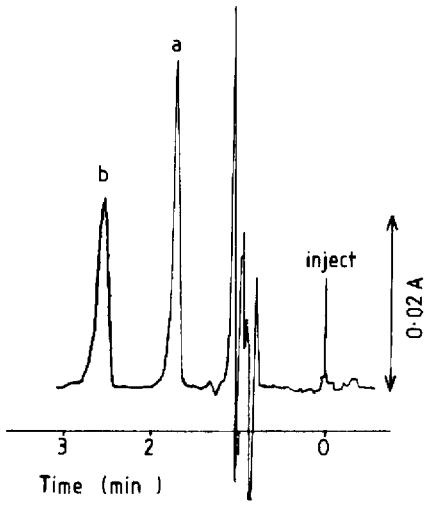

Fig. 3. Chromatogram obtained following incubation of adrenaline with caeruloplasmin under the conditions described. Peaks: (a) adrenochrome; (b) adrenaline.

oxidation of each amine at various substrate concentrations were determined by spectrophotometric measurement of the aminochrome produced. The results for adrenaline and 5HT are shown in Fig. 4. Noradrenaline and dopamine behaved in a similar fashion to adrenaline. The Michaelis constant, K_M, was calculated for each amine oxidation by using reciprocal plots of these data. The K_M values obtained were adrenaline, $3.8 \cdot 10^{-4}$ M; noradrenaline, $3.4 \cdot 10^{-4}$ M; dopamine, $4.2 \cdot 10^{-4}$ M; and 5HT, $6.2 \cdot 10^{-3}$ M.

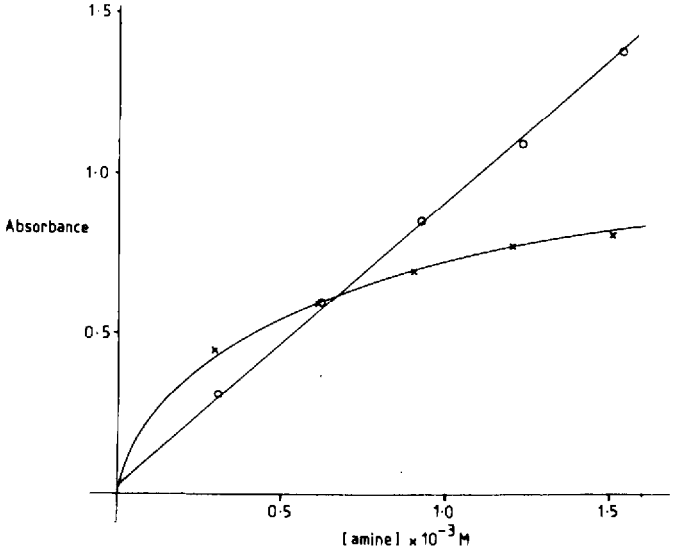

Fig. 4. Relationship between amine concentration and oxidation product formation at a fixed level of caeruloplasmin. Oxidation of adrenaline (×) was monitored at 300 nm; oxidation of 5-hydroxytryptamine (○) was monitored at 315 nm. Incubation conditions as described in the text.

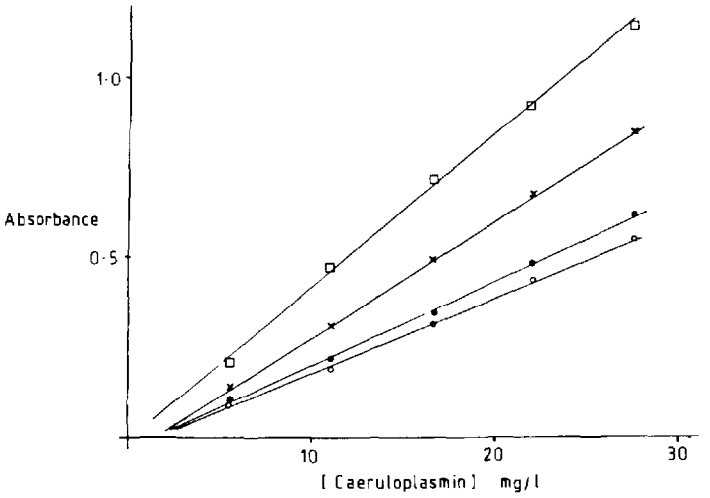

Fig. 5. Relationship between rate of oxidation (measured spectrophotometrically) and caeruloplasmin concentration for adrenaline (×), noradrenaline (○), dopamine (●) and 5-hydroxytryptamine (□). Incubation conditions as described in the text.

The substrate concentrations used in the above assay procedure were chosen so that the oxidations proceeded at the maximum rate. Under these conditions the rate of oxidation was linear with respect to time for 45 min for each amine.

The oxidation rate was also directly proportional to the enzyme concentration under the conditions of the assay (Fig. 5).

The within-batch reproducibility was determined by measuring the oxidation of each amine twenty times. The pooled control sera and purified caeruloplasmin included with each batch of sera analysed were used to calculate between-batch reproducibility. These results are shown in Table I.

Sera from twenty apparently healthy laboratory staff were incubated with each of the four amines and the oxidative activity was determined. The results obtained are summarized in Table II. The caeruloplasmin concentration of each sample was de-

TABLE I

PRECISION OF THE ASSAYS

Compound	*Precision*			
	Within-batch		*Between-batch*	
	No.	*C.V. (%)**	*No.*	*C.V. (%)**
Adrenaline	20	2.3	14	2.4
Noradrenaline	20	1.4	10	4.6
Dopamine	20	4.3	16	4.0
5-Hydroxytryptamine	20	3.9	8	2.8

* Coefficient of variation.

TABLE II

OXIDATIVE ACTIVITY OF NORMAL PLASMA SAMPLES

One unit of 5HT activity is defined as that amount of oxidation product formed by a calibrated 250 mg/l solution of caeruloplasmin in 45 min under the conditions of the assay described in the text.

	Adrenaline (*nmol min*$^{-1}$ *ml*$^{-1}$)	*Noradrenaline* (*nmol min*$^{-1}$ *ml*$^{-1}$)	*Dopamine* (*nmol min*$^{-1}$ *ml*$^{-1}$)	*5-Hydroxytryptamine* (*units*)
Mean	2.77	1.63	1.75	1.17
Standard deviation (S.D.)	0.70	0.42	0.37	0.29
Mean ± 2 S.D.	1.37–4.17	0.79–2.47	1.01–2.49	0.59–1.75

termined by immunodiffusion, producing a sample mean (n = 20) of 346 mg/l with a standard deviation of 81 mg/l. Comparison of these values with the measured oxidative activity gave correlation coefficients of 0.936 for adrenaline, 0.973 for noradrenaline, 0.959 for dopamine and 0.977 for 5HT.

The rate of oxidation of 5HT by standard solutions of purified caeruloplasmin was in agreement with that expected from the regression equation obtained with sera. However, catecholamine oxidation by purified enzyme proceeded at a rate that was around three times faster than that predicted by regression analysis of serum data.

DISCUSSION

The procedures described allow the determination of caeruloplasmin activity by monitoring the oxidation of naturally occurring substrates as opposed to the highly chromogenic, non-physiological substrates employed in many spectrophotometric assays. Having established the nature of the oxidation products by the use of difference spectra, it was noted that their stability was strongly influenced by pH and temperature. At pH greater than 6.0, the aminochromes rapidly broke down to produce a substance with a maximum absorbance at 390 nm. This is most likely to be the compound found to be produced in increased amounts by the sera of schizophrenics after oxidation of adrenaline[9], when the incubation was carried out at pH 7.4. In the assay procedure described the pH was maintained at 5.5, and it was shown that the aminochrome levels decreased by less than 5% in 6 h at this pH, provided that the tubes were stored on ice. This permitted the simultaneous incubations of large batches of amine oxidations, which could then be stored under the desired conditions prior to HPLC.

The K_M values obtained were comparable to those reported previously for caeruloplasmin using spectrophotometric or polarographic techniques[10,11].

The choice of an internal standard to compensate for variations in injection volume is made more difficult in enzyme assays by the additional criteria that the compound selected must not affect the enzyme reaction in any way. The use of the substrate as an internal standard, as described in this paper, would seem to be an ideal solution provided that (a) the substrate concentration greatly exceeds the concentration of product formed during the reaction period, and can therefore be assumed to remain constant, and (b) the detection characteristics of substrate and product are sufficiently different to permit optimal detection of low concentrations of product

with a comparable signal from the much higher concentrations of substrate, detected sub-optimally.

The application of the described assays to the study of the plasma enzyme was complicated by the presence of proteins in sufficient concentration to have a detrimental effect on the chromatography column if injected directly. Removal of plasma proteins by organic solvents or heavy metal ion precipitation could not be achieved without breakdown of the aminochromes. Therefore, centrifugal ultrafiltration was chosen as this had no effect on aminochrome stability, and permitted large numbers of samples to be processed in a short period of time.

The excellent correlations between the amine oxidation rates and the serum caeruloplasmin levels, determined immunologically, demonstrate that naturally occurring substrates can be used to measure plasma enzyme activity, and indeed are preferable to the commonly used aromatic amines, some of which are carcinogenic.

It is proposed to examine caeruloplasmin from the plasma of depressives to see if it behaves any differently towards the biogenic amines than the control group already studied, and this will be reported later.

Finally, it has been suggested that caeruloplasmin may have more than one binding or catalytic site for the biogenic amines[11], with 5HT occupying a different site to the catecholamines. The observation that 5HT oxidation by plasma caeruloplasmin proceeds at the rate expected from purified enzyme studies, whilst oxidation of catecholamines proceeds at only a third of the expected rate, supports the concept of separate sites for these compounds. If caeruloplasmin does play a role in the regulation of biogenic amine levels within the brain, then there may be interactions between these sites. It would therefore be of interest to study the behaviour of caeruloplasmin in the presence of more than one biogenic amine. This would not be possible using existing polarographic or spectrophotometric techniques as these would be unable to distinguish between the products of the different amines. However, modification of the HPLC method described would permit monitoring of the oxidation of amine mixtures, and this will be described in a further paper.

REFERENCES

1 C. G. Holmberg and C. B. Laurell, *Acta Chem. Scand.*, 2 (1948) 550.
2 E. Frieden and H. S. Hsieh, *Exp. Med. Biol.*, 74 (1976) 505.
3 B. C. Barrass and D. B. Coult, *Biochem. Pharmacol.*, 21 (1972) 677.
4 B. C. Barrass and D. B. Coult, *Biochem. Pharmacol.*, 22 (1973) 2897.
5 S. I. Rapoport and K. D. Pettigrew, *Microvasc. Res.*, 18 (1979) 105.
6 R. H. Henry, N. Chiamori, O. J. Golub and S. Berkman, *Amer. J. Clin. Pathol.*, 34 (1960) 381.
7 R. D. Kobes, R. J. Wyatt and L. M. Neckers, *J. Pharm. Methods*, 3 (1980) 305.
8 K. Rahman, T. Nagatsu and T. Kato, *J. Chromatogr.*, 221 (1980) 265.
9 B. E. Leach and R. G. Heath, *AMA Arch. Neurol. Psychiatry*, 76 (1956) 444.
10 B. C. Barrass, D. B. Coult, R. M. Pinder and M. Skeels, *Biochem. Pharmacol.*, 22 (1973) 2891.
11 B. C. Barrass, D. B. Coult, P. Rich and K. J. Tutt, *Biochem. Pharmacol.*, 23 (1974) 47.