Purification and Properties of Esterases Characteristic of Adult Rat Brain

By DANICA DABIČH, BHARGAVAN CHAKRAPANI* AND FRANK N. SYNER†

*Department of Biochemistry, Wayne State University School of Medicine
and Lafayette Clinic, Detroit, Mich. 48207, U.S.A.*

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1. A method for the partial purification of an esterase fraction, present in the brain of the adult but not the newborn rat, is described. A 54-fold purification was achieved in three steps. 2. When subjected to starch-gel electrophoresis, the purified fraction resolved into three bands of esterase activity. Two of these bands migrate close together and faster than other esterases in the brain. These two esterases are inhibited by *p*-hydroxymercuribenzoate but not by di-isopropyl phosphorofluoridate. The third band is di-isopropyl phosphorofluoridate-sensitive and migrates just behind the two leading esterases. 3. After treatment with di-isopropyl phosphorofluoridate, to obviate the effects of the di-isopropyl phosphorofluoridate-sensitive esterase, the enzyme preparation hydrolyses α -naphthyl acetate, α -naphthyl propionate and α -naphthyl butyrate, but not cholesteryl acetate. The $V_{\max}$ for the naphthyl esters decreased with increase in chain length of the acyl group. The acetate ester is hydrolysed 34 times as fast as the butyrate and about seven times as fast as the propionate derivative. The K_m values for these three esters, measured at pH 7.2 and 37°, are $2.8 \times 10^{-4} M$, $3.1 \times 10^{-4} M$ and $7.3 \times 10^{-5} M$ for the acetate, propionate and butyrate derivatives respectively. 4. The Hofstee (1952) plots for the kinetic data show a single line, indicating that the two most-rapidly migrating esterases, although electrophoretically separable, are not kinetically distinguishable in the substrate ranges examined.

A good index of the stage of development of the brain is the electrophoretic distribution of esterases on starch gels (Barron, Bernsohn & Hess, 1963; Bernsohn, Barron, Hess & Hedrick, 1963; Bernsohn, Barron & Norgello, 1964). When α -naphthyl acetate is used as substrate, 14–15 esterase bands are found on zymograms (Hunter & Markert, 1957) of brain extracts from animals of various ages. Only eight of these isoenzymes are readily demonstrable in brain extracts of newly born animals. Four other esterases, which are barely detectable on zymograms during the first 10 days *post partum*, increase rapidly during the subsequent 10–20-day period; these enzymes reach maximal activity 40 days after birth. Two additional esterases, which migrate anodically more quickly than any of the aforementioned ones, are characteristic of adult rat brain tissue (Bernsohn *et al.* 1963; Lagnado & Hardy, 1967). These isoenzymes are not visible on zymograms of brain extracts of animals less than

10 days old and are the ones with which this paper is concerned.

During the period from 2 to 20 days after birth, brain development in the rat is characterized by a slowing of growth rate and the beginning of myelin deposition (Sugita, 1918). Not only do brain proteolipids and cerebroside increase at this time, but the amount of free cholesterol in myelin, in contrast with cholesterol esters, increases (Folch-Pi, 1957). To ascertain whether the activity of the esterase isoenzymes unique to adult brain tissue relates to these changes a purified preparation of the enzymes was necessary. A method for purification of these isoenzymes is therefore described in this paper, as are measurements of pH optima, substrate specificity and effects of inhibitors.

MATERIALS

Hydrolysed starch, prepared by the Connaught Laboratories (Toronto, Ontario, Canada), tris and α -naphthol were purchased from Fisher Scientific Co., Detroit, Mich., U.S.A. The α -naphthyl acetate, propionate and butyrate derivatives, 2-mercaptoethanol, Triton X-100, sodium deoxycholate and *p*-hydroxymercuribenzoate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Cholesteryl

* Present address: Neurochemistry Research Department, C.M.C. Hospital, Vellore, India.

† Present address: Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Mich. 48207, U.S.A.

acetate was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Diamox (acetazolamide) was supplied by the American Cyanamid Corp., Stamford, Conn., U.S.A. Maleic acid was obtained from the Distillation Products Industries, Rochester, N.Y., U.S.A., and DFP* from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. CM-Sephadex was supplied by Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.

METHODS

Enzyme preparation. Adult Sprague-Dawley rats weighing 250–350 g. were used. All procedures were performed at 0–5° unless indicated to the contrary. A typical preparation is described below.

The animals were anaesthetized with ether and perfused by continuous infusion of cold 0.9% NaCl through the left ventricle of the heart after the jugular veins were severed. When the lungs had turned white and the fluid flowing from the jugular veins was free of blood, the animals were decapitated. The brains were removed and weighed as quickly as possible. Generally ten animals were used. The brains, weighing about 17.5 g., were transferred to a cold Potter-Elvehjem homogenizer, and 4 ml. of cold 0.9% NaCl, containing 0.2% (w/v) Triton X-100, was added/g. of wet tissue. The tissue was homogenized for 5 min. with a fairly tight-fitting Teflon pestle rotating at approx. 600 rev./min. A sample (2 ml.) of the homogenate was removed for electrophoresis and the determination of total esterase activity and protein concentration as described below.

The homogenate (85 ml.) was centrifuged at 20000g for 20 min. The clear supernatant solution was carefully decanted and a 2 ml. sample was removed for analysis.

After measurement of the volume of the supernatant solution (55.5 ml.), 390 mg. of $(\text{NH}_4)_2\text{SO}_4$ was added/ml., to give 60% saturation (Green & Hughes, 1955). After 1 hr. the proteins that were precipitated were removed by centrifugation for 15 min. at 20000g. The supernatant was brought to 70% saturation by the addition of 69 mg. of $(\text{NH}_4)_2\text{SO}_4$ /ml. of this solution. After 1 hr. the solution was centrifuged as above. The proteins that sedimented were dissolved in 5 mm-tris-phosphate buffer, pH 6.5, containing 1 mm-2-mercaptoethanol. The solution was dialysed overnight against the same buffer, which was changed several times, before chromatography as below.

CM-Sephadex C-25 was allowed to swell and was washed

according to the manufacturer's directions. The pH of the slurry, in water, was adjusted to 6.5 before three washes with 5 vol. of the buffer described above. Dialysed enzyme (3 ml.) containing about 2 mg. of protein/ml. was applied to a column (1.3 cm. $\times$ 30 cm.) of the CM-Sephadex, and was eluted with a linear ionic strength gradient formed by using 200 ml. of each of the following buffers in a mixing device. The starting buffer was identical in composition with that used for washing the resin; the buffer in the reservoir was the same, except that sufficient NaCl was added to bring its concentration to 1.0 M before adjustment of the pH of the solution to 6.5 with phosphoric acid.

Fractions (2 ml.) were collected at the rate of 24 ml./hr. The eluates were assayed for protein by the method of Warburg & Christian (1941) and for esterase activity according to the procedure described below. The enzymically active fractions were pooled and concentrated by pressure dialysis in collodion bags. The concentrated enzyme preparation was used for the studies outlined below. The yield and specific activity of the enzyme after each purification step are shown in Table 1.

Starch-gel electrophoresis. Vertical starch-gel electrophoresis was performed by the procedure of Smithies (1959), except that the starch was brought to 70–75° and never above 75°. The gel buffer was 0.02 M-borate, pH 8.4, and the bridge buffer 0.3 M-borate, pH 8.1. Electrophoresis was allowed to proceed at 4 v/cm. for 18 hr. in a cold-room (5°).

After electrophoresis the gel was cut in half longitudinally. One half of the gel was stained with Amido Black to locate the protein bands (Smithies, 1955). The other half was used for detection of the esterase activity, by the zymogram technique of Hunter & Markert (1957).

Assay for esterase activity in solution. A modification, described below, of the method of Gomori (1953) for assaying esterase activity in solution was used. The pH of the reaction mixture was 7.2.

The reagents for the enzyme assay include: 0.05 M-tris-maleate buffer, pH 7.2 (Gomori, 1953); 0.6 mm- α -naphthyl acetate substrate in buffer and 0.48 mm- α -naphthol in buffer. Control and standard solutions were prepared with each series of samples assayed. The control tube contained 2 ml. of substrate and 0.5 ml. of buffer. The tubes with α -naphthol standards contained 2 ml. of substrate and 0.2–0.5 ml. of α -naphthol. The test samples were a mixture of 2 ml. of substrate and 0.5 ml. of enzyme. When less than 0.5 ml. of enzyme solution or standard α -naphthol solution was used, sufficient buffer was added to bring the final volume to 2.5 ml. The tubes were incubated at 37° for 1 hr. and transferred to an ice bath. To each tube 2 ml. of

* Abbreviation: DFP, di-isopropyl phosphorofluoridate.

Table 1. *Purification of esterases unique to adult rat brain*

For details see the text.

Procedure	Vol. (ml.)	Total activity units (μ moles/min.)	Protein (mg./ml.)	Sp. activity (μ moles/min./mg. of protein)	Yield (%)
1. Homogenate of 17.5 g. of tissue	85	84	13.2	0.08	100
2. 20000g supernatant	55.5	43	8.0	0.10	51
3. $(\text{NH}_4)_2\text{SO}_4$ 60–70%-saturation fraction	3.8	4.3	3.7	0.31	5
4. Chromatography on CM-Sephadex followed by concentration	2.0	3.4	0.4	4.3	4

5-chloro-*o*-toluidinediazonium chloride solution (containing 200mg. in 100ml. of an aqueous solution of 0.5% Triton X-100) was added. The tubes were shaken well and kept at room temperature for 10min. The volumes were adjusted to 10ml. with water and the extinction at $500m\mu$ was measured. The esterase activity was expressed as μ moles of α -naphthol liberated/min. at 37° .

The above procedure also applied when α -naphthyl propionate and α -naphthyl butyrate were used as substrates. Tris-maleate buffer was used at pH values 8.5 and below. At higher pH values 0.05M-tris-HCl buffers (Gomori, 1955) were used.

Cholesteryl esterase activity of the enzyme preparation was tested by a modification of the method of Seshadri Sastry & Ganguly (1961). A homogeneous suspension of cholesteryl acetate was prepared as follows: 1ml. of cholesteryl acetate solution (30mm. in acetone) was diluted to 50ml. with 0.1M-tris-HCl buffer, pH 7.4, containing 0.2%

Triton X-100, and mixed well. The incubation mixture contained 1.2μ moles of cholesteryl acetate and 0.5ml. of enzyme preparation (or 0.5ml. of buffer for the blank) in a total volume of 2.5ml. The mixture was incubated at 37° for 12hr., and the cholesterol liberated was extracted with light petroleum (b.p. $30-60^\circ$) and determined by the method of Zak, Dickerman & White (1954).

Protein determinations. The procedure of Lowry, Rosebrough, Farr & Randall (1951) was used to measure protein concentration; when 2-mercaptoethanol interfered a modification of the Lowry procedure described by Moore & Lee (1960) was used.

RESULTS

Effect of detergents and organic solvents on the solubilization of esterases. Experiments with various detergents and organic solvents showed that Triton

Fig. 1. Electrophoretogram showing fractionation of rat brain esterases by $(\text{NH}_4)_2\text{SO}_4$. S represents the 20000g supernatant obtained as described in the text. The numbers below refer to the percentage saturation of $(\text{NH}_4)_2\text{SO}_4$ (Green & Hughes, 1955) at which the indicated esterases are precipitated; thus 20 refers to material precipitated between 0% and 20% saturation with $(\text{NH}_4)_2\text{SO}_4$. All other fractions were obtained by increasing the saturation by 10% increments. The numbers to the left indicate different α -naphthyl acetate esterases found in adult brain. The isoenzymes represented by numbers 1 and 2 are unique to adult rat brain tissue.

Fig. 2. Electrophoretogram demonstrating the effect of inhibitors on the brain esterase product from CM-Sephadex. (a) Control sample to which no inhibitor was added and which was developed according to the procedure of Hunter & Markert (1957). (b) Inhibition of the contaminating isoenzyme (3) with 0.1 mM-DFP. (c) Inhibition of the esterases 1 and 2, characteristic of adult brain, with 0.1 mM-*p*-hydroxymercuribenzoate. The concentration of adult-type esterases was sufficient to cause bands 1 and 2 to appear as a single broad band rather than as two discrete bands as shown in Fig. 1.

X-100 was most effective in increasing the yield of soluble esterases in the 20000g supernatant. At a concentration of 0.2% (w/v) of this detergent, 55% of the activity present in the homogenate was soluble compared with 12% in its absence; the specific activity was increased from 0.06 to 0.11 μ mole of α -naphthol liberated/min./mg. of protein. However, at 0.5% and 1%, the enzyme activity was inhibited by 20% and 30% respectively. With deoxycholate the optimum concentration was 0.06%, but only a 28% increase in soluble esterases was achieved with no increase in specific activity.

Use of 0.2% Triton X-100 appears to be as effective as repeated freezing and thawing (Bernsohn *et al.* 1963).

Ammonium sulphate fractionation. The stepwise fractionation of the 20000g supernatant with ammonium sulphate was monitored by starch-gel electrophoresis (Fig. 1). The fast-moving esterases, designated 1 and 2 in Fig. 1, are characteristic of adult brain tissue. These esterases are precipitated between 60% and 70% ammonium sulphate saturation; insignificant amounts of these esterases also appear in the fraction precipitated between 70% and 80% ammonium sulphate saturation. The strong bands appearing in Fig. 1 for this last fraction are misleading for, unlike the other fractions shown, it represents a solution that was concentrated before electrophoresis. The yield of esterase precipitated between 60% and 70% ammonium sulphate was, nevertheless, surprisingly low; it was approx. 4% of the total esterase activity present in the original brain extract.

Chromatography. Most of the esterase activity passed through the CM-Sephadex without adsorption, although an increase in specific activity always resulted (Table 1). The yields of esterase activity varied from 64% to 95% for a number of columns. Electrophoretic analysis of the enzymically active eluents (Fig. 2a) revealed the presence of bands that would correspond to numbers 1, 2 and 3 in Fig. 1. Attempts to improve the separation of the isoenzymes by using a lower pH, e.g. 5.5, resulted in the loss of all enzymic activity. On the other hand, DEAE-cellulose chromatography yielded only esterases characteristic of the foetal brain (bands 4, 9 and 10 in Fig. 1).

Inhibitors. The isoenzymes characteristic of adult rat brain were inhibited by 0.1 mM-*p*-hydroxymercuribenzoate (Fig. 2). They were not affected by 1 mM-DFP or by 0.1 mM-Diamox. The latter compound is an effective inhibitor of carbonic anhydrase, which behaves as an esterase on starch gels when α -naphthyl acetate is used as substrate (Shaw, Syner & Tashian, 1962). An isoenzyme, represented by band 3 in Fig. 2, contaminated the esterase preparation; however, it was DFP-sensitive. The enzyme preparation was therefore treated with 0.1 mM-DFP before subsequent investigations.

Optimum pH. The pH optimum of the fractionated isoenzymes was found to be between 7.05 and 7.47 when α -naphthyl acetate was used as substrate (Fig. 3). When a 20000g supernatant solution that contained an entire spectrum of esterases (Fig. 1, column S) was used as the enzyme source, a similar pH optimum was found.

Substrate specificity. Four compounds were investigated: α -naphthyl acetate, α -naphthyl propionate, α -naphthyl butyrate and cholesteryl

Fig. 3. Effect of pH on the hydrolysis of α -naphthyl acetate by esterases unique to adult brain tissue. Details of the assay are described in the text. The esterase activity is expressed as μ moles of α -naphthol liberated/min./mg. of protein.

Fig. 4. Hofstee plot for the hydrolysis of α -naphthyl acetate by the isolated esterases. The reactions were performed at pH 7.2 and at 37° according to the procedure described in the text. The velocity is expressed as μ moles/min./mg. of protein. The substrate concentration ranged from 0.04 to 0.8 mM.

Table 2. K_m and V_{max} values of purified esterases (see Table 1)

The results were obtained by plotting data according to Hofstee (1952).

Substrate	K_m (M)	V_{max} . (μ moles/min./mg.)
α -Naphthyl acetate	2.8×10^{-4}	7.55
α -Naphthyl propionate	3.1×10^{-4}	1.08
α -Naphthyl butyrate	7.3×10^{-5}	0.22

acetate. Only the α -naphthyl derivatives were hydrolysed by the purified isoenzymes. The maximum velocity, V_{max} , and apparent K_m values (Table 2) were obtained from Hofstee (1952) plots (Figs. 4–6) for the naphthyl derivatives. The K_m value for the butyrate derivative was found to be significantly different from the other two substrates. In addition, the V_{max} value for α -naphthyl acetate is 34 times that of the corresponding butyrate derivative and about seven times that of the propionate homologue. This optimum reaction with α -naphthyl acetate was also clearly evident on starch gels.

The Hofstee plots are presented since linearity of Lineweaver–Burk plots over a relatively short range of substrate concentrations can be misleading (Dowd & Riggs, 1965). In addition, the Hofstee plot is especially useful if more than one enzyme is

Fig. 5. Hofstee plot for the hydrolysis of α -naphthyl propionate by the isolated esterases. The reactions were performed at pH 7.2 and at 37° according to the procedure described in the text. The velocity is expressed as μ moles/min./mg. of protein. The substrate concentration ranged from 0.04/0.8 mM.

suspected in the preparation (Hofstee, 1952). Because, in Figs. 4–6, straight lines were obtained rather than two intersecting lines with different slopes, the esterases of adult brain, although electrophoretically separable, are kinetically indistinguishable by these methods in the range of substrate concentrations tested.

Fig. 6. Hofstee plot for the hydrolysis of α -naphthyl butyrate by the isolated esterases. The reactions were performed at pH 7.2 and at 37° according to the procedure described in the text. The velocity is expressed as μ moles/min./mg. of protein. The substrate concentration ranged from 0.033 to 0.4 mM.

Because of the rapid hydrolysis of α -naphthyl acetate, it was somewhat surprising that the enzyme preparation was not capable of hydrolysing cholesteryl acetate. All the cholesteryl esterase activity of the brain was recovered in a fraction that was precipitated between 0% and 40% ammonium sulphate saturation, and it was inhibited by 0.1 mM-DFP.

DISCUSSION

The zymograms of rat brain esterases on starch gels show that the esterase pattern changes markedly with time, and new bands appear and others disappear as the animal matures (Bernsohn *et al.* 1964; Lagnado & Hardy, 1967). In contrast, the changes in kidney and liver appear to be mainly quantitative with respect to particular esterase bands present in the young animal (F. N. Syner & B. Chakrapani, unpublished work). The origin of the new bands in brain may result from (1) the activity of different genes in the older animal, (2) changes in cell type, e.g. the transition from neural to glial cells, or (3) changes in mobility caused by increased binding of substances, e.g. lipids, to the original molecules. The last-named process is conceivable, for a number of

esterases present in the brain of younger animals show the same substrate specificity as the esterases described here and are inhibited by *p*-hydroxy-mercuribenzoate but not by DFP. These esterases do, however, have electrophoretic mobilities different from those of their counterparts in the adult.

Although the esterases represented by bands 1 and 2 in Fig. 1 increase in concentration with time and appear to parallel the increase in free cholesterol found in myelin, the present investigations show that these enzymes do not have cholesteryl esterase activity, at least with cholesteryl acetate. The natural substrate and function of these esterases, which represent only a small proportion of the total tissue activity, therefore still remain obscure.

V_{max} and K_m values were obtained from Hofstee (1952) plots for the naphthyl derivatives. The fact that no breaks occurred in these plots shows that, unlike liver esterases (Stotz, 1955), the two isoenzymes characteristic of adult brain tissue are kinetically indistinguishable under the conditions used. On starch gels, also, these isoenzymes appear to have similar substrate specificity.

The two isoenzymes unique to adult tissue thus show the same developmental pattern and enzymic properties and, so far, are distinguishable only by their electrophoretic mobilities. More physical and chemical studies are required to examine the question whether these two represent aggregates of different sizes or whether there are differences in primary structure.

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REFERENCES

- Barron, K. D., Bernsohn, J. & Hess, A. R. (1963). *J. Histochem. Cytochem.* **11**, 139.
- Bernsohn, J., Barron, K. D., Hess, A. R. & Hedrick, M. T. (1963). *J. Neurochem.* **10**, 783.
- Bernsohn, J., Barron, K. D. & Norgello, H. (1964). *Biochem. J.* **91**, 24c.
- Dowd, J. E. & Riggs, D. S. (1965). *J. biol. Chem.* **240**, 863.
- Folch-Pi, J. (1957). In *Ultrastructure and Cellular Chemistry of Neural Tissue*, p. 121. Ed. by Waelsch, H. New York: Academic Press Inc.
- Gomori, G. (1953). *J. Lab. clin. Med.* **42**, 445.
- Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 144. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Green, A. A. & Hughes, W. L. (1955). In *Methods in Enzymology*, vol. 1, p. 76. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Hofstee, B. H. J. (1952). *Science*, **116**, 329.
- Hunter, R. L. & Markert, C. L. (1957). *Science*, **125**, 1294.
- Lagnado, J. R. & Hardy, M. (1967). *Nature, Lond.*, **214**, 1207.

- Lowry, O. H., Rosebrough, N. J., Farr, H. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Moore, B. W. & Lee, R. H. (1960). *J. biol. Chem.* **235**, 1359.
- Seshadri Sastry, P. & Ganguly, J. (1961). *Biochem. J.* **80**, 397.
- Shaw, C. R., Syner, F. N. & Tashian, R. E. (1962). *Science*, **138**, 31.
- Smithies, O. (1955). *Biochem. J.* **61**, 629.
- Smithies, O. (1959). *Biochem. J.* **71**, 585.
- Stotz, E. (1955). In *Methods in Enzymology*, vol. 1, p. 657. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Sugita, N. (1918). *J. comp. Neurol.* **29**, 119.
- Warburg, O. W. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
- Zak, B., Dickerman, R. C. & White, E. G. (1954). *Amer. J. clin. Path.* **24**, 1307.