Acta Chemica Scandinavica B 32 (1978) 727—732

Purification of Acid Proteinases from Calf Brain

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The purification was begun with acetone precipitation of minced brain tissue with subsequent proteinase extraction with 0.2 M sodium formate buffer (pH 3.5), reprecipitation with acetone and dialysis. Chromatographic separation on Sephadex G-200, DEAE-Sephadex A-50 and CM-cellulose was carried out in that order. Upon ion-exchange chromatography multiple forms of acid proteinases emerged; two of them were obviously identical with cathepsin D (EC 3.4.23.5), and two of them exhibited properties of cathepsin B (EC 3.4.22.1).

In the brain – as in other tissues – the proteins are in a state of continuous turnover.[1–3] Cerebral proteolytic enzymes may thus play an important role by regulating the breakdown of proteins according to the metabolic and physiological requirements of brain tissue. It has emerged from the studies of several workers that brain tissue contains a whole family of proteinases with widely differing pH optima.[3] Within the weakly acidic pH range, at about pH 3.0 – 4.0, cerebral proteolytic activity is highest.[4] The cerebral acid proteinases are highly concentrated with many other hydrolytic enzymes in lysosomes.[5] From these organelles they may become liberated in certain degenerative brain diseases, producing damage to cell integrity.[6] The exact physiological role of brain acid proteinases has not yet been settled however, although they have been characterized to some extent.[7–8] In the present study we have purified acid proteinase components from calf brain and studied some of their enzyme properties.

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RESULTS

Acid proteinase preserved its activity well during acetone precipitation and the subsequent extraction of the dried acetone powder with formate buffers at low temperatures. A considerable proportion of proteins were more strongly retained by the Sephadex G-200 gel than the acid proteinase (Fig. 1). The recovery of total proteinase activity was very good up to this purification step (Table 1). In DEAE-Sephadex A-50 ion exchange chromatography the proteinase activity was separated into two distinct peaks I and II (Fig. 2) which were subsequently individually applied to a CM-cellulose column. In this column, peak I yielded two distinct proteinase peaks (Fig. 3), the first one being eluted with the plain 0.02 M sodium acetate buffer (fraction A) and the second one at about 0.04 M NaCl (fraction B). The second DEAE-Sephadex A-50 peak II likewise yielded two activity peaks in the CM-cellulose column (Fig. 4). The first was again eluted with the sodium acetate buffer (fraction C), while the second emerged at about 0.08 M NaCl (fraction D).

Purification varied from 194- to 278-fold among the proteinase fractions A to D obtained (Table 1). The most effective step in the whole purification procedure was the extraction of the dried acetone powder with 0.2 M sodium formate buffer, which resulted in a nearly tenfold increase in the specific activity of the proteinase accompanied by only an insignificant loss in total activity. About one third of the original activity in tissue homogenates was ultimately recovered in the four fractions together. Approximately one third of the activity was lost in both the preparation of the

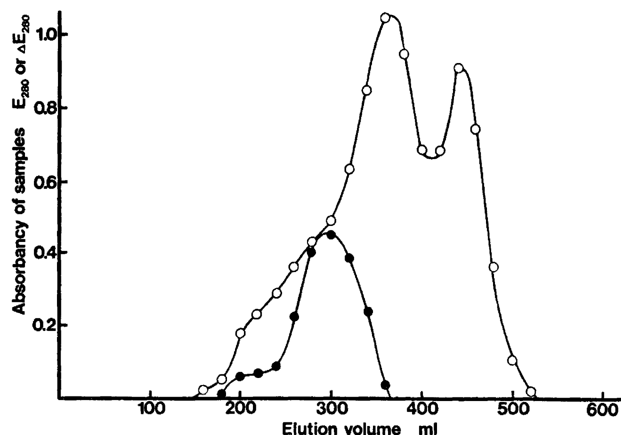

Fig. 1. Chromatogram of calf brain acetone powder in a Sephadex G-200 column (2.0 cm × 90 cm, 280 cm^3) in 0.05 M Tris-HCl buffer (pH 7.8, flow rate 15 ml/h). Acid proteinase activity (ΔE_{280}, ●) and protein content (E_{280}, ○) of the eluent fractions.

acetone powder and ion exchange chromatography on DEAE-Sephadex A-50. The purified proteinase was very stable in prolonged storage, losing only 30 % of the original activity after five months at 253 K in a Tris-HCl buffer of pH 7.8. In the disc electrophoresis of the purified proteinase preparations in polyacrylamide gels at pH 8.9 in Tris-glycine buffers only one protein band was visible.

In all fractions A, B, C and D the pH curves of the proteinase activity are suggestive of two pH optima, at pH 3.0 – 3.2 and at about pH 4.2 – 4.4 (Fig. 5). The proteinase activity in fractions A and C was unaffected by 0.1 mM 4-chloromercuribenzoate, and 10 mM 2-mercaptoethanol caused no significant activation either. On the other hand, the proteinase activity in fractions B and D was significantly inhibited by 4-chloromercuribenzoate and enhanced by 2-mercaptoethanol.

Of the divalent cations tested, Ca^{2+} was generally slightly activating, whereas Cu^{2+} and Cd^{2+} were inhibitory to various degrees (Table 2). Fraction C was least affected and fractions B and D most affected by these agents. No catheptic activity when tested with

Table 1. Purification of the acid proteinase from the calf brain.

Purification step	Total protein content mg	Total proteinase activity arbitrary units	Specific proteinase activity arbitrary units/mg protein	Purification coefficient	Recovery %
Brain homogenate	14600	2628	0.18	1	100
Acetone powder	6100	1830	0.30	1.6	70
Acetone powder extract	730	1825	2.50	13.8	69
Sephadex G-200 eluent	154	1617	10.5	58	62
DEAE-Sephadex A-50 eluent					
– peak I	16.6	266	16	89	10
– peak II	30.4	683	22	125	26
CM-cellulose eluents					
– fraction A	3.1	108	35	194	4.1
– fraction B	2.0	92	46	256	3.5
– fraction C	7.4	370	50	278	14.1
– fraction D	7.3	292	40	222	11.1

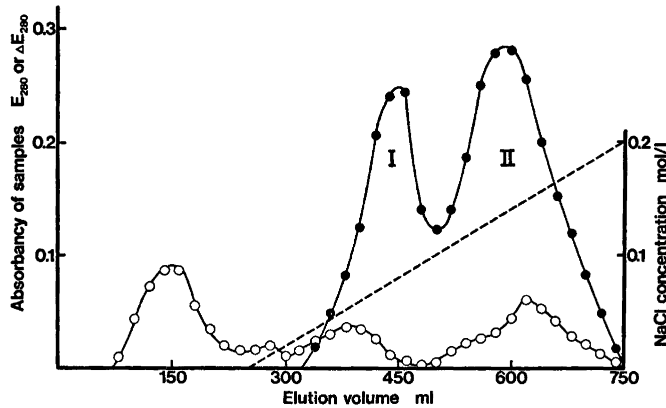

Fig. 2. Chromatogram of the acid proteinase peak obtained from the Sephadex G-200 column (Fig. 1) in a DEAE-Sephadex A-50 column (3.2 cm x 37 cm, 296 cm^3) in 0.05 M Tris-HCl buffer (pH 7.8, flow rate 30 ml/h) with a linearly ascending NaCl gradient (dashed line from 0 to 0.2 M). Acid proteinase activity (ΔE_{280}, ●) and protein content (E_{280}, ○) of the eluent fractions. The two proteinase peaks I and II were separately rechromatographed as shown in Figs. 3 and 4.

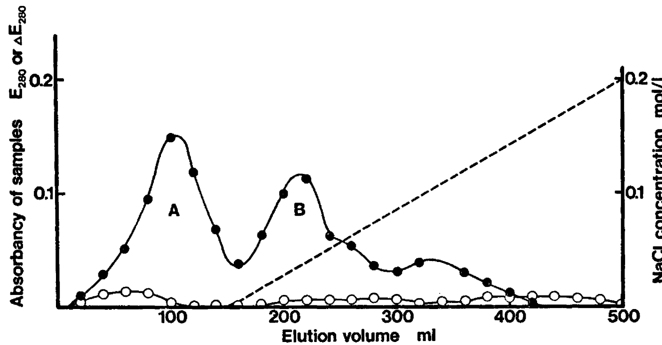

Fig. 3. Chromatogram of the acid proteinase peak I obtained from the DEAE-Sephadex A-50 column (Fig. 2) in a CM-cellulose column (1.5 cm x 18 cm, 32 cm^3) in 0.02 M sodium acetate buffer (pH 5.5, flow rate 15 ml/h) with a linearly ascending NaCl gradient (dashed line from 0 to 0.2 M). Acid proteinase activity (ΔE_{280}, ●) and protein content (E_{280}, ○) of the eluent fractions. The proteinase fractions A and B were used for further studies.

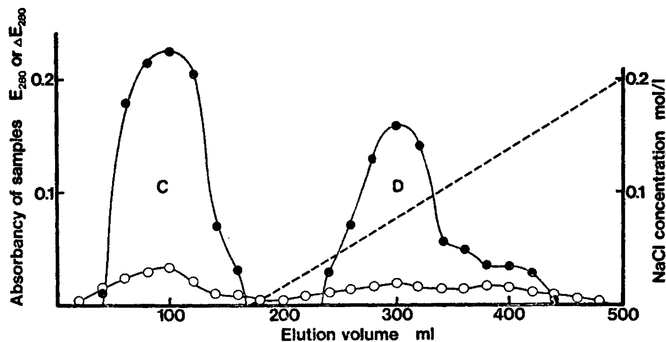

Fig. 4. Chromatogram of the acid proteinase peak II obtained from the DEAE-Sephadex A-50 column (Fig. 2) in a CM-cellulose column. Experimental details as in Fig. 3. Acid proteinase activity (ΔE_{280}, ●) and protein content (E_{280}, ○) of the eluent fractions. The proteinase fractions C and D were used for further studies.

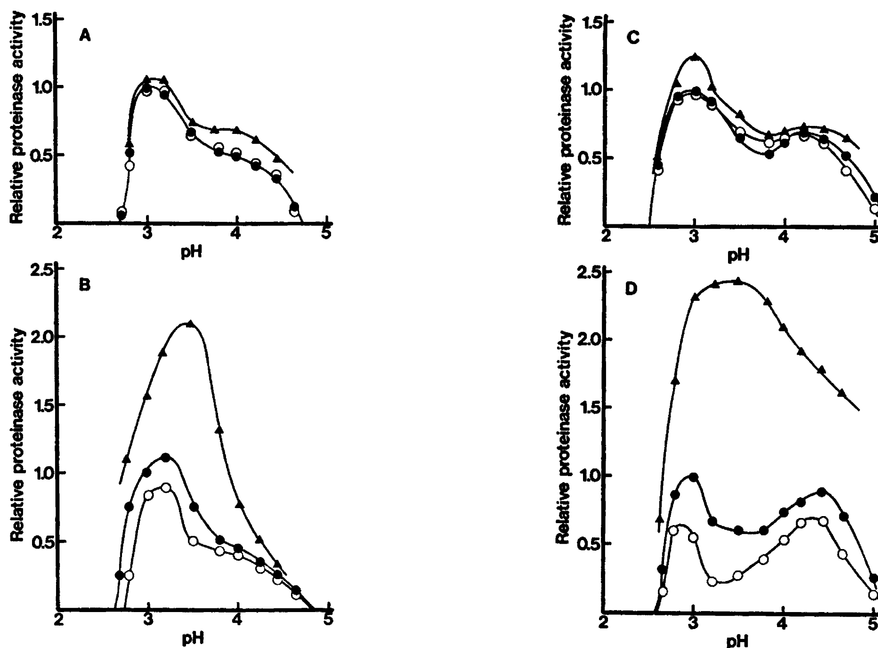

Fig. 5. pH dependency of the purified acid proteinase fractions A, B, C and D in 0.2 M glycine-HCl (pH 2.0 – 3.0) or sodium acetate (pH 3.0 – 6.5) buffers in the presence of 0.1 mM 4-chloromercuribenzoate (○) or 10 mM 2-mercaptoethanol (▲) or in the absence of these effectors (●).

BPANE could be found in any fractions. All the tests with the tryptic substrate, BAPA, were likewise negative. A weak activity towards the other tryptic substrate, BANA, was discernible at pH 7.5 in fractions B and D, particularly after extension of incubation up to 22 h at 310 K.

Table 2. Effects of Ca^{2+}, Cd^{2+} and Cu^{2+} ions on the acid proteinase activity in the enzyme fractions obtained [a].

Fraction	Ca^{2+}	Cd^{2+}	Cu^{2+}
A	$+4\pm8$	-14 ± 4*	-25 ± 6**
B	$+7\pm1$**	-22 ± 4**	-28 ± 2**
C	$+8\pm1$**	-5 ± 3	0 ± 4
D	$+25\pm7$*	-12 ± 2**	-38 ± 6**

[a] Numbers indicate the percentage increment or decrement (±S.E.M.) in acid proteinase activity at pH 3.5 in the presence of 1 mM Ca^{2+}, Cd^{2+} and Cu^{2+} when compared to controls without cations. Number of determinations is six. Significance of differences from controls: * $P<0.05$, ** $P<0.01$.

DISCUSSION

The purification yielded several acid proteinase fractions, of which four were more precisely characterized. The properties of two of the fractions (A and C) seem to be identical to the known properties of cathepsin D (EC 3.4.23.5).[9] Enzyme activity in these fractions was not sensitive to divalent metal ions and thiol group-blocking reagents like 4-chloromercuribenzoate. Neither was the activity enhanced by 2-mercaptoethanol. A division of cathepsin D into several partially separable subfractions upon ion-exchange chromatography or free-flow electrophoresis has also been observed on other occasions.[8,10] The catalytic properties of these multiple enzyme forms are obviously identical.[9] The pH dependency curves of all purified proteinase fractions were peculiar in showing more or less marked shoulders at pH 4.0 – 4.6 in addition to the main pH optimum at a still lower pH range. This bimodal characteristic is an inherent propensity of cathepsin D itself,[11] and does not indicate the existence of other contaminating

proteinases or isoenzyme heterogeneity. We may thus assume that fractions A and C both represent brain cathepsin D without major contaminants of other tissue proteinases.

Thiol groups seem to be involved in the proteinase activity in fractions B and D, as there was an inhibition provoked by the organomercurial compound and an enhancement by 2-mercaptoethanol. Fractions B and D also showed some activity towards the synthetic proteinase substrate BANA. The catalytic properties of fractions B and D resemble those of cathepsin B (EC 3.4.22.1), the presence of which has been verified in a number of other tissues,[12] although the cathepsin B activity has previously been assumed to be rather low in the brain.[8] Some of the cathepsin B activity in tissue extracts may also originate from the proteinase activity in the plasma or cellular elements of the blood trapped in the vascular bed of excised tissues.[3]

Like cathepsin D, cathepsin B also yields multiple enzyme forms upon ion-exchange chromatography with high resolution.[13–14] There is then a possibility for cross-contamination of the purified cathepsin B and D subfractions with each other. If there is more cathepsin D than cathepsin B in brain tissue, this would signify that the present proteinase fractions B and D should have contained a substantial amount of cathepsin D as well. The two-topped pH curves also in fractions B and D in the absence of 2-mercaptoethanol may be a sign of this contamination, although there are no reference data on the pH dependence of cathepsin B isolated from other tissues with respect to its action on the protein substrates. The use of pepstatin, a newly discovered specific pepsin and cathepsin D inhibitor, would probably be decisive in differentiating between the purified cathepsin B and D activities.[15–16] Both cathepsins B and D are intracellularly localized in lysosomes and a clear demarcation as to their individual physiological roles has not been feasible. A more manifold purification than that attained here would surely be possible after initial separation of lysosomes from other cell organelles.

EXPERIMENTAL

Bacto hemoglobin (Difco Laboratories), denaturated with urea,[17] was used as substrate in acid proteinase assays. The enzyme activity was measured by Kunitz's method.[18] The standard reaction mixtures contained 0.2 ml of 2 % (w/v) hemoglobin substrate solution, 0.8 ml of 0.2 M sodium acetate buffer (pH 3.5) and 0.2 ml of enzyme solution. When the pH was varied, 0.2 M glycine-HCl buffer was used within the pH range from 2.0 to 3.0, 0.2 M sodium acetate buffer from 3.0 to 6.5 and 0.2 M Tris [tri(hydroxymethyl)aminomethane]-HCl buffer from 6.5 to 10.5. In certain experiments the hemoglobin substrate solution contained divalent cations or thiol reagents to give final concentrations of 1 mM $CaCl_2$, $CuCl_2$ and $CdCl_2$ 0.1 mM 4-chloromercuribenzoate or 10 mM 2-mercaptoethanol.

The incubation time was 1 h at 310 K. The reaction was stopped with 1.5 ml of 10 % (w/v) trichloracetic acid solution in water, the resulting protein precipitate centrifuged down and the extinction of the supernatant measured at 280 nm. In the control (zero) samples the hemoglobin substrate solution was added after the trichloracetic acid solution. The extinction values of the control samples were subtracted from those of the corresponding test samples. These differences (ΔE_{280}) served as estimates of the acid proteinase activity. $\Delta E_{280}/(1\ \text{h})(1\ \text{mg})$ is used as an arbitrary estimate of the specific proteinase activity. A linear relationship was obtained between ΔE_{280} and the proteinase activity when ΔE_{280} was less than 0.3.

The presence of any tryptic activity in the samples was assayed by following enzymatic liberation of 2-naphthylamine from α-*N*-benzoyl-DL-arginyl-2-naphthylamide (BANA) and 4--nitroaniline from α-*N*-benzoyl-DL-arginyl-4--nitroanilide hydrochloride (BAPA). Chymotryptic activity was tested by measuring the liberation of 2-naphthol from α-*N*-benzoyl-DL-phenylalanyl-2-naphthyl ester (BPANE). The 4-nitroaniline liberated from BAPA was directly measured spectrophotometrically at 410 nm and the 2-naphthylamine liberated from BANA and 2-naphthol from BPANE after a diazo coupling reaction[19] at 525 and 540 nm, respectively. The incubation time varied up to 22 t, the temperature was 310 K and pH either 3.2, 4.2 or 7.5. The incubation mixture contained 1.0 ml of 1 M sodium acetate buffer (pH 3.2 and 4.2) or Tris-HCl buffer (pH 7.5), 0.5 ml of enzyme solution and 0.5 ml of 4 mM BANA, BAPA or BPANE solution in 90 % (v/v) methanol in water. At the end of incubation 0.5 ml of 1 M sodium acetate buffer (pH 4.2) in water containing 2 g/l Fast Garnet GBC and 100 g/l (w/v) Tween-20 was added to the samples containing BANA or BPANE, and these samples were incubated for a further 10 min at 310 K before spectrophotometry.

Protein determinations were according to Lowry *et al.* (1951), using bovine albumin as a reference. Distribution of protein in chromatographic effluents was checked by matching their extinctions at 280 nm, E_{280}, against the buffer

solutions used. Disc-electrophoresis of the purified proteinase preparations was done with Shandon's apparatus in 7.5 % (w/v) polyacrylamide gels in 0.3 M Tris-HCl buffer, pH 8.9[20]. The electrode solution was 0.2 M Tris-glycine buffer, pH 8.9. The gels were stained with 1 % (w/v) Amino Black 10 B in 0.9 M acetic acid.

Acid proteinases were purified six times from calf whole brains using about 200 g brain for each purification procedure. Acetone powder was first prepared from brain tissue as modified from Morton.[21] Brain tissue was thoroughly minced in 10 volumes of cold acetone at 262 K. The resulting precipitate was filtered out after 15 min and rinsed twice with five volumes of cold acetone. It was dried in a cold air stream and stored at 273 K in a vacuum exsiccator over a desiccant.

Acid proteinase was extracted from the acetone powder with 12 volumes of 0.2 M sodium formate buffer (pH 3.5) in water which contained 20 % (v/v) acetone, vigorously mixing the suspension for 1 h. It was then centrifuged at 10 000 *g* for 20 min. The precipitate was discarded and the supernatant slowly poured into 4 volumes of cold (262 K) acetone under mixing and then centrifuged again as above. The supernatant was now discarded and the precipitate drained and then suspended with 1.5 volumes of 0.05 M Tris-HCl buffer (pH 7.8) in water. The suspension was vigorously mixed for 30 min and centrifuged at 10 000 *g* for 15 min. The supernantant was separated and the precipitate was washed with 0.5 volumes of the above Tris-HCl buffer and centrifuged as before. The precipitate was discarded and both supernatants were combined and dialyzed against Tris-HCl buffer for 18 h at 277 K.

The dialyzed enzyme extract was applied to a Sephadex G-200 column (2.0 cm × 90 cm, 280 cm^3) equilibrated with the above Tris-HCl buffer. The column was eluted with 500 ml Tris-HCl buffer (15 ml/h). Fractions of 5 ml were continuously collected and a sample of 0.2 ml was taken from each fraction for assays of their proteinase activity and protein content. The fractions showing proteinase activity were combined (altogether 70 ml in volume) and applied to a DEAE-Sephadex A-50 column (3.2 cm × 37 cm, 296 cm^3) equilibrated with the above Tris-HCl buffer. The column was first eluted with 250 ml Tris-HCl buffer (30 ml/h) and then with a linear NaCl gradient (from 0 to 0.2 M) in 750 ml Tris-HCl buffer. Fractions of 6 ml were continuously collected. A sample of 0.2 ml was taken from each fraction for assays of proteinase activity and protein content. The fractions belonging to two partially distinct proteinase peaks were separately combined and dialyzed against 0.02 M sodium acetate buffer (pH 5.5) in water for 18 h at 277 K. The two dialyzed proteinase fractions were separately applied to CM-cellulose columns (1.5 cm × 18 cm, 32 cm^3) equilibrated with the above sodium acetate buffer. The columns were first eluted with 150 ml sodium acetate buffer (15 ml/h) and then with a linearly ascending NaCl gradient (from 0 to 0.2 M) in 350 ml sodium acetate buffer. Proteinase activity and protein content of the collected fractions of 5 ml were assayed as above. All the above column chromatographic steps were carried out at 277 K.

REFERENCES

1. Oja, S. S. *Ann. Acad. Sci. Fenn. A. V. 131* (1967) 1.
2. Lajtha, A. and Marks, N. In Lajtha, A., Ed., *Handbook of Neurochemistry*, Plenum, New York 1971, Vol. 5 B, Chapter 3.
3. Marks, N. and Lajtha, A. In Lajtha, A., Ed., *Handbook of Neurochemistry*, Plenum, New York 1971, Vol. 5 A, Chapter 2.
4. Oja, S. S. and Oja, H. *J. Neurochem. 17* (1970) 901.
5. Marks, N. and Lajtha, A. *Biochem. J. 89* (1963) 438.
6. Einstein, E. R. In Lajtha, A., Ed., *Handbook of Neurochemistry*, Plenum, New York 1972, Vol. 7, Chapter 6.
7. Palladin, A. V., Polyakova, N. M. and Lishko, V. K. *J. Neurochem. 10* (1963) 187.
8. Marks, N. and Lajtha, A. *Biochem. J. 97* (1965) 74.
9. Barrett, A. J. In Barrett, A. J., Ed., *Proteinases in Mammalian Cells and Tissues*, North-Holland, Amsterdam 1977, Chapter 5.
10. Press, E. M., Porter, R. R. and Cebra, J. *Biochem. J. 74* (1969) 501.
11. Cunningham, M. and Tang, J. *J. Biol. Chem. 251* (1976) 4528.
12. Barrett, A. J. In Barrett, A. J., Ed., *Proteinases in Mammalian Cells and Tissues*, North-Holland, Amsterdam 1977, Chapter 4.
13. Suominen, J. and Hopsu-Havu, V. K. *Acta Chem. Scand. 25* (1951) 2531.
14. Otto, K. and Riesenkönig, H. *Biochim. Biophys. Acta 379* (1975) 462.
15. Marks, N., Grynbaum, A. and Lajtha, A. *Science 181* (1973) 949.
16. Takahashi, K., Chang, W.-J. and Ko, J.-S. *J. Biochem. 76* (1974) 897.
17. Bergmeyer, H.-U. *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim 1962, p. 808.
18. Umaña, R. *Anal. Biochem. 26* (1968) 430.
19. Riekkinen, P. J. and Hopsu, V. K. *Ann. Med. Exp. Fenn. 43* (1965) 6.
20. Smith, M. E. *Biochim. Biophys. Acta 164* (1968) 285.
21. Morton, R. K. In Colowick, S. P. and Kaplan, N. O., Eds., *Methods in Enzymology*, Academic, New York 1955, Vol. 1, p. 25.

Received May 29, 1978.