A Comprehensive Study of the Postnatal Changes in the Concentration of the Lipids of Developing Rat Brain*

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ABSTRACT: The concentration of 24 classes of lipids of brain was determined for 3-, 6-, 12-, 18-, 24-, 42-, 180-, and 330-day-old rats. On the basis of these data, correlations were made between the appearance and rate of deposition of lipids with known cytological changes. Sterol ester decreased to undetectable levels within 6 days after parturition while ganglioside immediately increased but continued to increase markedly only during the early stages of myelination. A group of lipids consisting of cerebroside, sphingomyelin, triphosphoinositide, phosphatidic acid, galactosyldiglyceride, and inositol plasmalogen occurred at concentrations less than 10% of that found in the adult brain up to the onset of myelination and then rapidly increased in concentration throughout the period of active myelination. Sulfatide showed a similar pattern of change except that it also increased markedly in concentration before myelination began. Ethanolamine plasmalogen, ethanolamine phosphoglyceryl ether, cholesterol, diphosphatidylglycerol, and phosphatidylserine occurred in concentrations between 17 and 34% of that found in the mature brain and showed moderate increases in concentration prior to myelination and increased markedly during myelination. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol occurred in concentrations 49–60% of that found in adult brain prior to myelination and their concentrations increased only moderately during subsequent development. The pattern of change of phosphatidylglycerol was intermediate to these two last groups of lipids. Phosphatidylglycerol phosphate increased in concentration up through the 12th day, remained fairly constant through the 42nd day, and then decreased to one-fifth the maximum level by the 180th day. No significant concentration of glycerides or serine plasmalogen was seen at any age.

One approach to an understanding of the physiological role of the various compounds of the brain and to their localization within particular structures of the brain is to study the changes that occur during development. This type of study of the lipids of rat brain was initiated by the work of Koch and Koch (1913). Many subsequent measurements have been made, all of which have been limited to a few of the lipids now known to occur in brain. For example, in the most comprehensive study made up to now, Brante (1949) was limited by the technical knowledge of the time to the determination of eleven different classes of rat brain lipids. The development of an analytical procedure for the quantitative determination of 24 different brain lipids (Wells and Dittmer, 1966) now makes it possible to present a comprehensive study of lipid changes in rat brain during growth and development.

Experimental Section

Virgin, male Sprague–Dawley rats from a single colony were used for this study except for 3- and 6-day-old rats which were not selected on the basis of sex. The rats were maintained on a standard diet. In general brains from six rats of each age were pooled and randomly divided into two samples and duplicate analyses were carried out on the extracts of each sample. With the 3-day-old rats, nine brains were analyzed in three samples and with the 330-day-old rats only four of the six rats being maintained for this group survived to this age and duplicate analyses were run on the extracts from two samples of two brains each. The brains were excised and extracted under conditions that minimized changes due to autolysis (Wells and Dittmer, 1965).

The analyses were carried out as described by Wells and Dittmer (1966). In order to handle the large number of samples involved in this study, eight analytical anion-exchange column fractionations of the mild alkaline and acid hydrolysates were run simultaneously. Each set of four columns was eluted with a single gradient prepared in three chambers of a Buchler Varigrad. The gradient was pumped with a Durrum Model 12AP, 12-channel chromatography pump.

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TABLE I: The Lipid Composition of Developing Rat Brain.

Lipid[a]	Concentration (μmoles/g wet weight)							
	3 Days	6 Days	12 Days	18 Days	24 Days	42 Days	180 Days	330 Days
Phosphoglycerides								
Phosphatidylcholine	14.72	14.82	20.38	24.38	24.79	24.95	24.65	24.89
Phosphatidylethanolamine	5.25	5.66	7.96	9.37	11.00	10.89	10.72	10.49
Phosphatidylglycerol	0.12	0.20	0.16	0.20	0.29	0.27	0.28	0.29
Phosphatidylinositol	1.21	1.38	1.59	1.86	2.04	2.17	2.20	2.30
Phosphatidylserine	2.91	3.56	4.51	6.10	7.04	8.25	8.50	8.97
Phosphatidylglycerol phosphate	0.10	0.13	0.20	0.16	0.16	0.17	0.06	0.04
Phosphatidic acid	0.14	0.21	0.26	0.39	0.70	1.03	1.31	1.36
Diphosphatidylglycerol	0.19	0.21	0.34	0.52	0.57	0.68	0.60	0.55
Diphosphoinositide	0.01	0.05	0.05	0.16	0.15	0.19	0.21	0.20
Triphosphoinositide	0.03	0.04	0.05	0.14	0.17	0.24	0.41	0.39
Choline plasmalogen	0.04	0.06	0.07	0.09	0.16	0.22	0.35	0.34
Ethanolamine plasmalogen	2.19	2.75	4.73	7.02	11.3	13.5	13.0	13.2
Inositol plasmalogen	0[b]	0	0.05	0.08	0.13	0.13	0.13	0.11
Plasmalogenic acid	0.02	0.05	0.15	0.17	0.18	0.15	0.20	0.12
Ethanolamine phosphoglyceryl ether	0.18	0.23	0.38	0.69	1.02	1.06	1.04	1.13
Sphingolipids	0.23	0.26	1.04	2.15	3.19	3.62	3.70	4.10
Sphingomyelin								
Cerebroside	0[c]	0	2.3	5.8	10.3	18.6	21.8	22.5
Sulfatide	0.14	0.32	0.79	1.28	2.04	3.22	4.22	4.48
Ganglioside								
Hexosamine	0.31	0.73	0.84	0.96	1.01	1.06	1.15	1.13
Sialic acid	0.51	1.27	1.45	1.74	1.97	2.08	2.18	2.07
Others								
Cholesterol	10.7	12.6	22.6	32.2	38.3	39.5	40.2	40.6
Galactosyldiglyceride	0.05	0.06	0.31	0.86	1.29	1.46	1.56	1.62
Brain weight (g)	0.40	0.61	1.11	1.34	1.55	1.63	1.85	1.86
Per cent recovery of phosphorus in combined fractions	97.0	96.5	98.0	98.5	98.0	97.0	97.0	98.0

[a] Data for sterol ester, glycerides, and serine plasmalogen are given in the text. [b] Lower level of detection would be on the order of 0.01 μmole/g. [c] Lower level of detection would be on the order of 0.05 μmole/g.

Fractionation of the alkali–acid-stable lipids was carried out by running four silicic acid columns simultaneously. The solvents were pumped through a manifold with a Milton Roy chromatography pump equipped with a stainless-steel liquid end.

Results

The analytical data for 21 different classes of lipids are given for the brains of 3-, 6-, 12-, 18-, 24-, 42-, 180-, and 330-day old rats in Table I. All analyses on all classes of lipid at each age agreed within 10%. In addition to these data it was shown that there was no significant amount of simple glyceride or serine plasmalogen in any of the brains analyzed. There was a significant amount of sterol ester in the 3-day-old rat brain, 2.0 μmoles/g wet weight. In all the other samples, the sterol ester was less than 5% of the free sterol present and significant data were not obtained.

Discussion

There has been considerable discussion in the past about the best mode in which to present data of this type, *e.g.*, MacIlwain (1959), and consequently a few words on this point are appropriate. The data have been interpreted primarily on the basis of two parameters that can be readily calculated from the concentrations given in Table I. In order to consider the over-all changes in concentration for any given lipid, the concentration at any particular age has been considered as the per cent of the concentration of the lipid in the brain of the 180-day-old rat. The relative change in concentration of different lipids during a short period

has been considered on the basis of the proportional increment in concentration during that period. Graphical presentation of the original data or the calculated parameters proved to be rather unwieldy.

The anatomical and histological observations with which the data obtained in this study are correlated are taken from the summary given by MacIlwain (1959). The development of the rat brain is conveniently considered in four periods. *Period I* encompasses the time up to birth and is characterized by differentiation and proliferation of cells. During this period, the number of cells in the rat brain reaches 94–97% of the adult number. We have no data for this period. *Peroid II* lasts from birth until approximately 10 days. In this period the brain increases in size and the outgrowth of axons and dendrites occurs. This period, represented in our study by the 3- and 6-day-old rats, involves changes that are associated primarily with the grey matter. Myelin is virtually absent during this time. *Period III* begins about the 10th day and lasts until the 20th day. During these 10 days the mass of the brain doubles and the water content decreases. Myelination begins and continues at a rapid rate throughout this period. Growth of cells continues but at a reduced rate. For the first time alterations in the electrical potential of the cortex can be detected and neuromuscular control begins. This phase of development is represented in our study by the 12- and 18-day-old rats. *Period IV* begins about the 20th day and lasts until full maturation of the brain is reached. Myelination continues during this time but at a decreased rate. This final stage is represented in our study by the 24-, 42-, and 180-day-old rats.

The data from this study indicate that the various lipids of rat brain can be grouped into five categories on the basis of correlations with the periods of development described above. Briefly, these include (1) lipids that are associated exclusively with active changes in the nonmyelinated brain, (2) lipids that are primarily associated with myelination, (3) lipids associated both with myelination and other major membrane structures apparently not directly related to myelination, (4) lipids having no association with myelination and showing only relatively small changes in concentration during development, and (5) lipids that show changes in concentration that cannot be correlated with recognized developmental changes or the changes overlap the first four groups. These correlations are summarized in Table II for the three periods studied.

The lipids of the first group (sterol ester and gangliosides) changed markedly in concentration only during the earliest phases of development. Sterol esters which were present in the brains of 3-day-old rats in significant concentration dropped by the 6th day to undetectable levels. These data on sterol esters agree with previous observations on the chick by Mandel *et al.* (1949) and on the chick and human by Adams and Davison (1959). The latter workers correlated the time at which the greatest proportion of sterol was present as the ester with the onset of myelination in both human cervical cord and corpus callosum. Because myelination does not begin in the rat until well after the 3rd day, we do not believe a similar correlation can be made for the rat. The pattern of change in the rat suggests that the sterol esters may have been associated with the proliferation of cells that is just ending at birth. This view is borne out by the observations of Gopal *et al.* (1963) and Slagel *et al.* (1967) that brain tumors in which cell proliferation but little or no myelination is occurring have a relatively high sterol ester content. No other lipid in rat brain had a pattern of change similar to that of the sterol esters.

Gangliosides showed a marked increase during period II that ended in period III. By the third day, the ganglioside concentration was 24% of the adult level and a 2.5-fold increase in concentration occurred by the 6th day. The concentration continued to increase until 90% of the adult level was reached by the 24th day. A similar pattern of change has been reported in the rat brain in several earlier studies (Pritchard and Cantin, 1962; James and Fortherby, 1963; Burton *et al.*, 1963; Kishimoto *et al.*, 1965). The substantial concentration at 3 days and the rapid rise in concentration only during the initial 24 days of the postnatal period are consistent with the view that gangliosides are associated primarily with the grey matter or perhaps more specifically with the cell growth that occurs during period II and ends in period III. The possibility of involvement of gangliosides in myelination cannot be ruled out since the last 30–40% increase in concentration occurred after myelination had begun. James and Fotherby (1963) have, however, reported that the gangliosides are found in highest concentration in tissue samples consisting predominately of grey matter.

The second group of lipids was characterized by low levels and little change in concentration during period II, a very rapid increase in concentration in period III, and a slower, continued increase in period IV. This pattern of change was consistent with the occurrence of these lipids primarily in myelin because myelin is virtually absent during period II and is rapidly formed in period III and then continues to be formed at a slower rate during period IV. Cerebroside, sphingomyelin, triphosphoinositide, phosphatidic acid, galactosyl diglyceride, and inositol plasmalogen comprise this group. They occurred at concentrations less than 10% of the adult level during period II. Between the 12th and 18th day (period III) these lipids showed a two- to threefold increase in concentration. During the early part of period IV (12–24 days) a 1.1- to 1.8-fold increase in concentration occurred followed between the 24th and 42nd day by an additional 1.1- to 1.8-fold increase. The data reported here agree with earlier values for sphingomyelin and cerebroside (*e.g.*, Brante, 1949; Bieth *et al.*, 1961; Freysz *et al.*, 1963) when allowance is made for the fact that the cerebroside values also generally include sulfatide and galactosyl diglyceride. The observed pattern of change and the fact that cerebroside and sphingomyelin are found in high concentrations in white matter have led to the widely accepted view that these are the most characteristic lipids of myelin.

TABLE II: Summary of Lipid and Morphological Changes.

Period	Age of Rats Studied (days)	Characteristic Morphological Changes	Description of Lipid Changes				
			Class 1[b]	Class 2[c]	Class 3[d]	Class 4[e]	Class 5[f]
II	3 and 6	Axons and dendrites grow out. Myelin is virtually absent.	Sterol ester disappeared. Ganglioside increased from 24 to 60% of adult level.	On 3rd day <10% the adult concentration found and little change occurred up to the 6th day.	Between 3rd and 6th day these lipids increased 1.2- to 1.7-fold from a range of 17 to 34% the adult level.	These lipids increased from a range of 49–60 to 60–63% the adult level between 3rd and 6th day.	Sulfatide and DPI[a] increased markedly between the 3rd and 6th day from less than 5% the adult level.
III	12 and 18	Rapid phase of myelination.	Ganglioside reached 73% of adult level by 12th day and increased to 83% by 18th day.	10–28% of the adult concentration reached on 12th day and a two- to three-fold increase occurred by the 18th day.	In general these lipids increased to from 36–53% the adult level by the 12th day and to 54–87% by the 18th day.	Concentrations continued steady increase with 84–99% of the adult level being reached by the 18th day.	Sulfatide and DPI increased 1.6- and 3.2-fold between 12th and 18th day. PGP[a] reached maximum concentration.
IV	24, 42, and 180	Decreased rate of myelination; maturation.	Ganglioside reached adult level by 24th day.	In general, increase continued from a range of 47–86% at the 24th day to 58–100% the adult level on the 42nd day. The exceptions (PA[a] and TPI[a]) showed greatest increase in last part of period.	Continued increase to concentrations of 82-98% the adult level by the 24th day; 97–100% of the adult level reached by 42nd day. Choline plasmalogen showed greatest increase in last part of period.	Adult levels reached by the 24th day.	Sulfatide and DPI increased in manner similar to class 2 lipids. PGP decreased in concentration to lowest levels found.

[a] Abbreviations used: DPI, diphosphoinositide; PGP, phosphatidylglycerol phosphate; TPI, triphosphoinositide; PA, phosphatidic acid. [b] Lipids that appear to be primarily associated with changes in the nonmyelinated brain. [c] Lipids that appear to be associated primarily with myelination. [d] Lipids apparently associated with changes in both myelinated and nonmyelinated brain. [e] Lipids that show no changes that can be directly related to morphological changes. [f] Lipids that show marked changes during development not directly associable with morphological changes.

Eichberg *et al.* (1964) do not concur with this view particularly with regard to sphingomyelin because of the distribution of these lipids found in isolated subcellular fractions of the guinea pig brain. Their conclusions in regard to sphingomyelin echo those of Nussbaum *et al.* (1963) who analyzed the phospholipids of the subcellular fractions of rat brain. Data for the distribution of phosphatidic acid and triphosphoinositide (Eichberg and Dawson, 1965) indicate that these lipids do occur predominately in myelin. No equivalent data for the distribution of inositol plasmalogen and galactosyl diglyceride are available.

The third group of lipids was present in period II in amounts which varied from 17 to 34% of the adult level, and showed a marked increase in concentration which started in period II and continued through periods III and IV. Ethanolamine plasmalogen, choline plasmalogen, diphosphatidylglycerol, ethanolamine phosphoglyceryl ether, cholesterol, and plasmalogenic acid comprise this group. Between the 3rd and 6th day (period II) all members of this group showed a 1.2- to 1.7-fold increase in concentration. In period III the concentration of these lipids increased at rates (1.3- to 1.8-fold increase between 12th and 18th day) that were generally lower than the lipids of the second group and higher than the lipids of the fourth group. The pattern of change of concentration of the lipids of the third group may be interpreted to mean that these lipids were associated with nonmyelin membrane structures that were actively undergoing change during period II. These same structures may have undergone continued change during periods III and IV or during these last two periods some or all of the change may have been associated with myelination. No distinction between the two possible explanations for the change during periods III and IV can be made on the basis of the temporal relationship.

The fourth group of lipids is comprised of those that were present at concentrations of 49–60% of the adult level during period II and showed only moderate changes in concentration throughout the period of development studied. This group includes phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Phosphatidylglycerol appeared to be intermediate between groups 3 and 4. The fourth group appeared to be only passively involved throughout development and were obviously of little significance in myelination.

With the exception of reports on cholesterol and total plasmalogen content of rat brain during development there is little directly comparable data available on the concentration of the lipids that have been singled out in the third and fourth groups. Our values fall within the limits of the range reported in previous studies of plasmalogens (Korey and Orchen, 1959; Erickson and Lands, 1959; Stammler *et al.*, 1954; Bieth *et al.*, 1961; Freysz *et al.*, 1963) and cholesterol (*e.g.*, Mandel and Bieth, 1951). The analysis of all the different plasmalogens has not been previously reported. Where data on the distribution of specific lipids between the white and grey matter of adult brain are available (*e.g.*, Brante, 1949; Johnson *et al.*, 1948), they are not inconsistent with the grouping given here. Plasmalogen, cholesterol, and phosphatidylserine are found in greater proportion in the white than in grey matter, and phosphatidylethanolamine and phosphatidylcholine are found predominately in the grey matter. The distribution of lipids in subcellular particles in the brains of guinea pig (Eichberg *et al.*, 1964) and rat (Nussbaum *et al.*, 1963) is only in part consistent with our interpretation. In the guinea pig there is an enrichment of ethanolamine plasmalogen, cholesterol, ethanolamine phosphoglyceryl ether, and phosphatidylserine in the myelin relative to the whole brain and a corresponding lower proportion in most of the nonmyelin fractions. On the other hand, Eichberg *et al.* found no diphosphatidylglycerol in the myelin fractions. Phosphatidylglycerol content is not reported. Data on phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol are not consistent within their study. "Large myelin fragments" were shown to have relatively low proportions of these lipids as would be expected from our data; however, "small myelin fragments" are enriched with each of these lipids relative to whole brain.

Phosphatidylglycerol phosphate, diphosphoinositide, and sulfatide did not fit into any of the four categories described above. Phosphatidylglycerol phosphate increased in concentration during period II, remained fairly constant through period III, and then finally dropped to from one-third to one-fourth its maximum level during period IV. No other lipid showed such a dramatic drop in concentration with maturation. This lipid has been implicated as an intermediate in the synthesis of phosphatidylglycerol in liver (Kiyasu *et al.*, 1963) and is also probably involved in diphosphatidylglycerol synthesis. If in analogy with most of the other phospholipids the rate of synthesis of phosphatidylglycerol and diphosphatidylglycerol is greatest during period II followed by a decline in period III, the increase in concentration of phosphatidylglycerol phosphate is consistent with it having an intermediary role in a pathway with a rate-limiting reaction subsequent to its formation.

The concentration of diphosphoinositide changed in a pattern similar to that observed for the second group of lipids except that it also increased in concentration during period II. Here again the difference may be attributed to a role as an intermediate in the synthesis of another lipid. Diphosphoinositide is a precursor of triphosphoinositide and the rise in concentration of diphosphoinositide precedes the rapid rise in concentration of triphosphoinositide during period III.

The low concentration of sulfatide in period II, 3–8% of the adult level, and the rapid rise in concentration during periods III and IV was similar to the pattern of change of the second group of lipids, that is, those lipids that appear to be primarily associated with myelination. It differs in that it also showed a significant increase in concentration during period II. In this respect the pattern of change was similar to diphosphoinositide or to the lipids in the third group;

however, the low initial concentrations distinguished it from the latter group of lipids. The data from previous studies on the change of concentration of lipid sulfate (Koch and Koch, 1913; Davison and Gregson, 1962; Bakke and Cornatzer, 1961) with age in rat brain show a similar pattern of change. It appears certain that like the lipids in the third group, sulfatide must be associated with both nonmyelin and myelin structures. The distribution of lipid sulfur in subcellular fractions of rat brain as determined by Davison and Gregson (1962) supports this interpretation. They estimate that approximately 50% of total lipid sulfur is in the myelin.

The value of this study lies in its comprehensiveness, for with few exceptions, the data are in good agreement with that from previous studies on rat brain. Data for over half the different individual classes of brain lipids have been obtained for the first time; and because the data for each class of lipids are directly comparable, it has been possible to make such distinctions as that made, for example, between the lipids that we have placed in the second, third, and fourth groups, and between the cerebrosides and sulfatides.

Some difficulty in interpretation arises from the fact that throughout development there is a loss of water and an increase in the concentration of protein and other constituents. Because of the loss of water, a lipid may show an increase in concentration even though the absolute amount of lipid, micromoles per brain, remains the same or decreases. The increase of other constituents will have the opposite effect, that is, the apparent concentration of any given lipid is decreased. The water loss can be compensated for by a consideration of concentrations based on dry weight but otherwise this mode of presentation has little or no advantage. With the criteria we have used to group the lipids, neither the decrease in water concentration nor the increase in concentration of other constituents cause any ambiguity, that is, although the absolute proportional increments and per cent of concentrations are dependent on these factors the relative values between lipids are independent of them. A more serious difficulty lies in the fact that the concentrations for the various lipids are not independent of each other. A large increase in the absolute amount of a given lipid will be reflected as a relative lower increase in the concentration of all the other lipids. No attempt to compensate for this has been made and in actual practice the use of a parameter such as the micromoles per brain which is for each lipid mutually independent was not found to be any more informative than the parameters used.

The correlation of changes in lipid concentration with cytological changes during development as a means of distinguishing the roles of the various lipids is subject to many limitations. The fact that a particular histological change appears to predominate during a given period of development does not rule out the possibility of changes in lipid composition in other structures. A further complication arises from the fact that all parts of the brain do not develop at the same rate nor is it certain that similar structures in all parts of the brain have the same lipid composition. These latter difficulties can, of course, be overcome by analyzing the different parts of the brain during development rather than the whole brain. The alternative or complementary approach of directly analyzing isolated brain cellular fractions is also subject to several limitations including the degradation of lipids and the possibility of redistribution of lipids between structural components during the time required to effect fractionation. In view of these considerations, it is perhaps surprising that there are no greater discrepancies between the interpretation which we place on our data and the data available from studies of the distribution of lipids in isolated fractions. With refinement of both techniques a fully compatible picture of the developmental cytological distribution of lipids should be possible.

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Acidic Bovine Pancreatic Trypsin Inhibitor. I. Purification and Physical Characterization*

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ABSTRACT: A polypeptide trypsin inhibitor has been isolated and purified from a 15% sodium chloride filtrate which is discarded during the commercial preparation of bovine insulin. The inhibitor was isolated by precipitation at 31% sodium chloride, reprecipitation with 0.6 saturated ammonium sulfate, and chromatography on CM-cellulose using pH 5.0 ammonium acetate buffers. The purified inhibitor behaved as a single component on acrylamide and starch gel electrophoresis. Column chromatography, gel electrophoresis, and amino acid content indicated that this inhibitor is more acidic and is distinctly different from the basic Kunitz pancreatic trypsin inhibitor.

The molecular weight of our inhibitor was found to be 6500 ± 300. Phenylalanine, tryptophan, and histidine were found to be absent. Our inhibitor is identical with an acidic pancreatic trypsin inhibitor isolated in much smaller quantities by Kazal *et al.* (Kazal, L. A., Spicer, D. S., and Brahinsky, R. A. (1948), *J. Am. Chem. Soc. 70*, 3034) from a similar side fraction, and later by Greene *et al.* (Green, L. J., Fackre, D. S., and Rigbi, M. (1966), *J. Biol. Chem. 241*, 5610) from pancreatic juice.

Kunitz and Northrup (1936) first crystallized a trypsin inhibitor from bovine pancreas. Kazal *et al.* (1948) isolated a crystalline trypsin inhibitor from a side fraction of the commercial bovine insulin process of Romans *et al.* (1940). Kazal's inhibitor differed from the Kunitz inhibitor in that it was found to be nondialyzable, and its ionic character was of a more acidic nature. The Kazal inhibitor was found to be electrophoretically heterogeneous. The crystalline inhibitor of Kazal was obtained in yields of 12–172 μg/kg of frozen pancreas. Haverback *et al.* (1960) and Keller and Allen (1967) found the Kazal-type inhibitor in human pancreatic juice. Fritz *et al.* (1966a–c) demonstrated the presence of a similar type of inhibitor in human, pig, cow, and dog pancreas and pancreatic juice. Grossman (1958) reported a Kazal-type inhibitor in rat pancreatic juice. Greene *et al.* (1966) found a trypsin inhibitor in bovine pancreatic juice and determined its amino acid content and physical characteristics. This paper presents a very short chromatographic procedure which gives an electrophoretically homogeneous pancreatic trypsin inhibitor in milligram quantities per kilogram of frozen bovine pancreas. The inhibitor isolated by us is identical with the inhibitor isolated by Kazal *et al.* (1948) from frozen pancreas and later by Greene *et al.* (1966) from pancreatic juice.

Materials and Methods

Assay. Trypsin esterase activity was assayed by the spectrophotometric method of Schwert and Takenaka (1955). The inhibitor was assayed by the method of Kassell *et al.* (1963) using the Determatubes TRY (Worthington Biochemical Corp.) as the substrate solution. Crystalline trypsin and crystalline KPTI[1] (Worthington Biochemical Corp.) were dried for 24 hr *in vacuo* over P_2O_5. Dried trypsin (8 μg) in the assay cuvet caused an increase in OD_{253} of 0.099/min. KPTI (1 μg) caused a decrease in the above ΔOD_{253} of 0.055/min. One unit of our inhibitor (APTI) activity is equivalent to the inhibitory activity of 1 mg of this standard KPTI. Specific activity is defined as units

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[1] Abbreviations used: KPTI, Kunitz pancreatic trypsin inhibitor; APTI, acidic pancreatic trypsin inhibitor; FDNB, fluorodinitrobenzene.