THE RIBONUCLEASE ENZYME SYSTEM IN RAT BRAIN

by

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B.A., University of British Columbia 1970

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the Department
of
Biochemistry

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
August 1975

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ABSTRACT

A study was made of the enzyme system responsible for the catabolism of ribonucleic acid in rat brain. Initial work with whole brain homogenates and extracts revealed the presence of three ribonucleases (RNases) distinguishable by the pH at which they exhibit optimal activity. The identified RNases are referred to according to their pH optima as pH 6.7 RNase, pH 7.8 RNase and pH 9.5 RNase. Evidence was also obtained indicating the presence of a protein inhibitor of the pH 7.8 RNase. The components of this multi-enzyme-inhibitor system were separated and partially purified by ammonium sulphate fractionation of whole brain extracts followed by DEAE-cellulose column chromatography of the ammonium sulphate precipitable fractions.

The DEAE-cellulose eluate RNases were characterized with regard to the effect of various reagents upon their activity. The intracellular distribution and developmental profiles of the three RNase activities and the pH 7.8 RNase inhibitor activity were also determined.

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ACKNOWLEDGEMENTS

I would like to express my immeasurable gratitude to Shan-Ching Sung for his limitless patience, trust, understanding and encouragement without which this work could not have been sustained or completed.

I would like to thank my laboratory colleagues Jen-Fu Chiu, Vijendra Singh, Raja Rosenbluth and Jim Nagy for their companionship, thoughtfulness and helpfulness.

I also thank all the people of the Kinsmen Laboratories of Neurological Sciences and the Health Science Centre Hospital, with whom I have shared so much time and space, feeling and thought, for the privilege of knowing them and through knowing them expanding the limits of my understanding and appreciation of the inner workings of the human brain.

Finally, I thank the many others who intersected my life in the course of this project for the inspiration they instilled in me and for all they have taught me.

It is only in collaboration with all of the abovementioned people that the ongoing *in vivo* processes of my own brain have been sustained and regulated throughout the course of this work.

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I. INTRODUCTION

1.0 General Frame of Reference

It is generally regarded[1-7] that tissue-specific differences in macromolecular composition are a consequence of the differential expression of genome information common to all somatic cells. The problem of formulating neurobiological phenomena in molecular terms is hence essentially that of understanding the specific nature, expression and regulation of the genetic capabilities of the nervous system and its constituent cell types.

Severe methodological difficulties arising from the organizational complexity and morphological heterogeneity of the mammalian central nervous system have hindered progress toward this goal and, consequently, the molecular understanding of brain function has lagged behind that of other organs.

Biochemical studies of the brain have followed four main avenues of approach. First, there have been studies concerned with elucidating those changes in molecular composition which produce or parallel morphological changes as cells differentiate toward their mature structural and functional states. Information as to the molecules and molecular events occurring in the course of development will permit a more detailed understanding of how they participate

to yield the specific morphology, metabolism, and functional capabilities of the fully differentiated cell types of the adult brain.

Secondly, considerable effort has been directed toward achieving an understanding of the molecular composition and metabolism of normal adult brain and correlating brain-specific molecular constituents with the unique morphology and functional characteristics of neuronal tissue.

Thirdly, attempts have also been made to detect alterations in molecular composition and molecular function accompanying normal adult changes in neuronal function. Morphological changes in fully-differentiated neurons have been demonstrated[8] to occur in response to changes in sensory input and behavior-modifying challenges from the external environment.

An understanding of the molecular events correlated with or responsible for such morphological alterations will help to elucidate the nature and limits of interaction between sensory experience and intracellular metabolism; that is, how perturbations of intraneuronal metabolism are effected, the capacity of the molecular mechanisms regulating metabolic adaptation and hence the limits of response ability of metabolic processes within fully-differentiated neuronal cell types. It is hoped that such work will eventually

result in the identification of those brain-specific molecules and molecular events which may mediate the processing and storage of sensory information or which may underlie cognitive and affective processes and other psychobiological phenomena peculiar to the central nervous system.

Finally, there have been studies of how alterations in the genome can affect defined functions of the nervous system in neurological and behavioral mutants; such studies can provide clues concerning the contributions made by single, identified gene products to complex and integrated brain function and behavior.

It has become clear from such studies that both the comparatively gross molecular changes which occur throughout development[1,3] and the more subtle neurochemical correlates of experientally- and behaviorally-related neural activity in the adult brain[9-16] entail quantitative and qualitative changes in the protein composition of cells.

These changes in cellular protein composition may occur within a particular cell, in a population of cells linked to one another structurally and functionally, and in even more complex systems. Proteins by their intervention as enzymes and membrane constituents influence all other molecular constituents of the cell. Hence, such changes in protein composition could be expected[17] to correspond

to the establishment of new functional connections between neurons, alterations in the properties of certain neurons, families of neurons, and in their steady-state mode of synaptic function[18-21].

Because ribonucleic acid is the primary gene product and any specific information manifested at this level of genome expression is subsequently transferred to proteins through the mechanism of translation (protein synthesis) to yield the tissue- and cell type-specific metabolism, morphology and functional capabilities of an organ, it seems likely that alterations in cellular protein composition may be secondary to changes in de novo RNA synthesis and RNA turnover.

Changes in cellular protein patterns thus arise as a consequence of modulations in gene expression--either directly through (1) changes in the rate of transcription of constitutively expressed genetic information, or through (2) qualitative shifts in the read-out of the reversibly expressible pool of genetic information (i.e., through induction or repression); or more indirectly by (3) post-transcriptional mechanisms regulating gene expression (modulation of RNA turnover, RNA translation and protein turnover). It is this third general category of regulation of cellular protein

composition which is the domain of concern specifically dealt with in the present thesis.

Prior to discussing the role of the enzyme system chiefly responsible for the post-transcriptional metabolism of RNA and the post-transcriptional control of cellular RNA content, it will be useful to review the information available to date on changes in content, composition and turnover of RNA in the infant and adult central nervous system.

1.1 Changes in Brain RNA During Ontogenesis

1.11 Changes in brain RNA content and composition during postnatal maturation

In the rat, total RNA content of whole brain increases up to the 15th to 18th day postnatally.[22,23,24] Subsequently, the nuclear and transfer RNA content[24,25] of whole brain remains relatively constant throughout adulthood whereas the microsomal and ribosomal RNA content declines.[24,26] There also occurs a change in the base composition--an increase in adenine and a commensurate decrease in guanine--of whole brain microsomal RNA as the brain matures.[26]

In the cerebral cortex of the rat, the increase in all classes of RNA ceases at 18 days and is followed by a 40% decrease in polysomal RNA by the time the animals reach

adulthood (200-250 g. body weight).[24] This decline in polysomal RNA content per cerebrum is accompanied by an increase in the pyrimidine and a decrease in the purine content of this RNA fraction, whereas the base compositions of nuclear RNA of cerebral cortex of both young and adult animals remains the same.[24]

Such developmental changes in the base composition of RNA isolated from the microsomal and polysomal fractions of brain are most likely accounted for in terms of (1) shifts in the base sequence and base compositions of specific mRNA molecules associated with ribosomes at different stages of development, and/or (2) an age-dependent increase in the mRNA/rRNA ratio in these fractions.

The ability of RNA molecules homologous in base sequence to compete in hybridization to complementary base sequences of DNA has been utilized to demonstrate alterations in the kind of genome information transcribed at different stages of ontogenesis.[27,31] Grouse et al.[32] have studied the ability of total RNA isolated from different regions of the adult mouse and human brain to compete with total RNA from the same brain regions of fetal or infant mouse and human brain hybridized to unique sequence DNA. They found that in both mouse and human brain there occurs with development

a net increase in the variety of transcribed RNA. Moreover, the greater transcriptional diversity evident in adult as compared to fetal brain is attributable to diversification of RNA in the cerebral cortex since the number of different kinds of RNA molecules present in other regions of the adult brain was similar to that of whole fetal brain. Twenty per cent of the maximal unique sequence genome information was expressed in whole adult brain compared to 6% for other organs in the human, and 12% as compared to 6% in the mouse. Similar competition hybridization studies[28] have indicated that in mouse liver the variety of mRNA molecules synthesized declines during development. Bondy and Roberts[33] demonstrated that during maturation chromatin prepared from whole rat brain as well as specific brain regions exhibits a decline in its capacity to function as a template for E. coli RNA polymerase-catalyzed RNA synthesis in vitro, indicating that during development there occurs a progressive template-limiting decline in the rate of transcription. Chromatin from brain was found to support the incorporation of more label into RNA than hepatic chromatin. The decline in template activity of chromatin was correlated with a concurrent decline in the non-histone protein content of chromatin. Such a developmental drop-out in non-histone chromosomal proteins associated with DNA has also been observed by Kurtz and Sinex[34]

in mouse brain and may be relevant to the explanation of the results of Grouse et al.[32] which indicate that chromatin from adult brain has more regions of its DNA derepressed and available for transcription. Bondy and Roberts[33] found that whereas the total mRNA transcribed from whole brain chromatin was lower in the adult than in the newborn rats, the mRNA fraction of the total RNA transcribed was proportionally higher in the adult than in the infant. Thus, although the total amount of mRNA-like RNA in rat brain also decreases during development,[35] the decrease in total rRNA is still greater and results in a net increase in the mRNA/rRNA ratio.

Bondy and Roberts[36] also found that nuclear RNA from both whole brain and liver of adult rats hybridized to rat total DNA to a greater extent (by about 20%) than cytoplasmic RNA. Both nuclear and cytoplasmic RNA from brain hybridized to a twofold greater extent than nuclear and cytoplasmic RNA from liver. The authors suggest that in the adult a larger proportion of RNA synthesis is directed towards mRNA production in brain as compared to liver. Such an inferrence is not unequivocally supported by the data since in this case the hybridization of RNA transcribed from single copy genes is not clearly distinguished from that of RNA which is the product of repetitive genes. Also, nuclei contain rapidly synthesized RNA molecules which are not transferred to the

cytoplasm[36,78] and this type of RNA presumably does not function as mRNA.

This conclusion is, nevertheless, supported by a considerable body of other evidence. Comparison of the base composition of RNA isolated from various subcellular fractions of brain and liver indicate that the adult brain contains more RNA which is DNA-like in its base composition than liver.[37] By this criterion brain has a greater nuclear heterodisperse RNA and mRNA content than liver.

Zomzely et al.[38] have also demonstrated that in the adult a larger proportion of RNA synthesis in vivo results in mRNA in brain as compared to other organs in the rat.

Whereas in brain the number of polysomes is relatively high at birth and declines during development,[39] in liver they are relatively few in number at birth and increase with development.[40] Concurrent with the decrease in polysomal RNA and in the total number of polysomes, as the brain matures there occurs a decrease in the number of membrane-bound polysomes[41] and a decrease in the size[25] and stability[41] of isolated polysomes.

It remains uncertain to what extent the smaller polysome size class distribution in the adult may represent (1) a developmental shift in the size of mRNA molecules associated with ribosomes[35,42] or (2) breakdown of polysomes during their

isolation[43] which may reflect age-related differences between the RNase activity levels of the post-mitochondrial supernatant from which polysomes are prepared. The proportion of polysomes to free ribosomes in brain has also been shown to be affected by sensory stimulation and to be dependent on the functional state of the tissue[25] (see section 1.3).

Adams[24] found that the rate of ^{14}C-orotic acid incorporation *in vivo* into nuclear RNA is the same for both the adult and 4-day old rat cerebral cortex. However, there is a lag period of about 60 minutes in the young animals which is not present in adults, during which labelling of the microsomal and ribosomal fractions of the cytoplasm proceeds only slowly.[24,39] Sharma and Singh[44] observed a similar lag in the rate of transfer of label from the nucleus to RNA in the cytoplasm in newborn rat whole brain slices incubated *in vitro*. Mandel[45] in studying developing chick brain observed that newly synthesized RNA remains within the nucleus of chick embryo neuroblasts and spongioblasts until about 15 days of embryonic life. The nature of the nucleocytoplasmic barrier responsible for this phenomenon is unknown. Murthy[39] has shown that the entry of polyribosomal RNA into the cytoplasm follows the temporal sequence: mRNA, 18S RNA, 28S RNA.

It is not known to what extent the rate-limiting step contributing to this difference is due to the rate of precursor RNA conversion to functionally mature RNA, the rate of RNA assembly into ribosomal subunits and informosomes or the rate of transport across the nuclear envelope.

Comparison of *in vivo* rates of RNA synthesis in the brain or specific brain regions of animals of different ages is complicated by the fact that intracranial routes of administering the labelled precursor have been demonstrated to result in uneven distribution of the label in different brain regions. Also, such studies have not controlled for age-dependent changes in the rate of uptake of label into the cells nor for differences in intracellular precursor pool size.

Guroff *et al.*[46] reported that the *in vitro* rate of ^{3}H-uridine incorporation/mg DNA was 3-fold greater in cerebral cortical slices than in cerebellar slices of the 10-day old rat. Between age 10 days and 4 months, the rate of RNA synthesis/mg DNA decreases 6-fold in the cortex and 10-fold in the cerebellum, so that the rate of RNA synthesis/mg DNA is about 6-fold higher in the adult cortex as compared to the adult cerebellum. Guroff also reported[46] that the rate of RNA synthesis/mg DNA was 2-fold higher in slices of liver than in slices of cerebral cortex of the adult rat.

Sung[47] found that the in vitro rate of ^{14}C-uridine incorporation/mg DNA in tissue slices incubated for one hour was slightly higher in the cerebellum than in the cortex of 2-day old rats. By age 10 days, the rate of RNA synthesis/mg DNA had decreased 2.2-fold in the cerebellum, whereas the cortex exhibited a 1.3-fold increase relative to the rate of RNA synthesis at age 2 days.

Johnson[48] studied the in vitro incorporation of ^{32}P-orthophosphate into RNA of mouse whol brain cell suspensions during a 2-hour incubation period and also found a rapid post-natal decline in the rate of RNA synthesis up to age 7 to 9 days.

Several workers have measured the rate of RNA synthesis in isolated nuclei incubated in vitro under conditions which preferentially detect either RNA polymerase A- or RNA polymerase B- catalyzed RNA synthesis.

Bondy and Waelsch[49,50] studied the incorporation of ^{14}C-UTP and ^{14}C-ATP into RNA of liver and brain cell nuclei incubated for 3 minutes in the presence of Mn^{++} and high ionic strength—conditions determined to be optimal for RNA polymerase B- catalyzed RNA synthesis. The rate of RNA synthesis/mg DNA under these assay conditions was consistently 2-fold higher for whole brain as compared to liver of rats at all ages between 8 days and 4 months. Moreover, the capacity

of nuclei for RNA polymerase B- catalyzed RNA synthesis increased almost proportionally in both organs during this time period resulting in a net increase of 20% for brain and 30% for liver. These workers also studied the regional distribution of RNA polymerase B- catalyzed RNA synthesis in the adult brains of the rabbit and squirrel monkey. Nuclei prepared from the cerebral cortex, caudate and hippocampus were found to exhibit 2-fold higher RNA synthesizing activity per mg DNA than those from the thalamus-hypothalamus, corpus callosum, pons-medulla and cerebellum.

Giuffrida et al.[51] studied the RNA synthesizing activity of cell nuclei prepared from the cerebral cortex, cerebellum and brainstem of 5, 10, and 18-day old rats. Utilizing assay conditions which preferentially discriminate between the rates of RNA synthesis catalyzed by RNA polymerase A and RNA polymerase B they found that under both conditions, nuclei isolated from all three brain regions of older rats exhibited substantially lower rates of ^{3}H-UTP and ^{3}H-GTP incorporation than those derived from the 5-day old rats. In 5-day old animals, nuclei from the cerebellum had a 1.4-fold higher rate of RNA polymerase B- catalyzed RNA synthesis than nuclei from both the cerebral cortex and brainstem, and underwent a 3.3-fold decrease to become 2-fold lower than the activity in the cerebral cortex and brainstem by 18 days. By

18 days the RNA polymerase B activity/mg DNA had declined in the cerebral cortex and brainstem to 75% and 86% respectively of their 5-day old level. RNA polymerase A- catalyzed RNA synthesis/mg DNA, assayed in the presence of Mg^{++} and low ionic strength, was slightly higher in the cortex and brainstem than in the cerebellum of 5-day old animals and underwent a 2.5-fold decrease in all three brain regions between 5 and 18 days.

Thus, there is a predominant amount of evidence indicating that on a per unit DNA and hence per nucleus basis, there occurs a decline in the rate of total cellular RNA synthesis as well as RNA polymerase A- and B- catalyzed RNA synthesis during development in the whole brain as well as in specific brain regions. On the basis of the consistent body of evidence it can be inferred that the demand for mRNA and rRNA, as indicated by the rate of RNA synthesis, is highest during cell division and cell differentiation and, subsequently, declines to lower levels as the brain matures and attains its adult number of cells by age 18 to 20 days.

However, the apparently anomalous results of Bondy and Waelsh[49,50] are not to be disregarded. These workers have reported finding an increase in the rate of RNA polymerase B-catalyzed RNA synthesis during maturation in all regions of the rabbit brain, in the whole brain of the rat and in the liver of both rabbit and rat.

The discrepancy between the results of different workers may be related to differences in the assay conditions for RNA polymerase B activity. Whereas Bondy and Waelsch's data were obtained with an incubation period of 3 minutes, Giuffrida et al.[51] have found that the Mn^{++}/$(NH_4)_2SO_4$ - stimulated reaction is linear for 45 minutes after an initial lag period of at least 5 minutes. This, however, does not offer an obvious explanation of the apparent discrepancy, since Barondes[53] in a study of Mn^{++}/$(NH_4)_2SO_4$ - stimulated RNA syntheis by "aggregate enzyme" during a similarly short incubation period of 5 minutes found a 2-fold lower rate of ^{14}C-CTP incorporation into RNA (per unit DNA) in the whole brain of 9-month old as compared to 12-day old rats. Also, in 9-month old rats, this enzyme preparation from brain was only 37% as active as that from liver.

The explanation for why the rate of RNA synthesis has been reported to be lower in brain as compared to liver during long incubation periods is not likely to be due to a more rapid degradation of newly synthesized RNA[54] in brain as compared with liver in view of the data reported by Dutton and Mahler.[55] These workers found that in adult rats the rate of RNA polymerase A- catalyzed RNA synthesis (Mg^{++} and low ionic strength) of nuclei prepared from the cerebral

cortex was only 2% of that of liver nuclei. They also found that the incorporation of ^{3}H-CTP into RNA was enhanced 25 to 50% by preincubation of brain nuclei at 37° (see similar observation by Guroff[46] for brain slices) whereas preincubation of liver nuclei for 30 minutes completely inhibited their capacity to synthesize RNA.[56] These workers concluded that the RNA synthetic capacity was more stable and the nuclear RNA more protected from nucleases in brain nuclei than in liver nuclei of adult animals.

Since results following brief incubation periods are probably more reliable measures of the initial rate of RNA synthesis and minimally affected by the rate of degradation of the newly synthesized product,[57] one would expect Bondy and Waelsch's data to be a more accurate measure of the *in vivo* rate of RNA synthesis than results obtained with longer incubation periods where the rate of degradation of the newly synthesized RNA becomes an important consideration, particularly if the rate of nuclear RNA turnover varies with age or shows tissue specific differences. However, the differential sensitivity of brain and liver nuclei to preincubation might also explain the discrepancy in results. If the duration between preparation of nuclei and assay of nuclear RNA synthesizing activity was long in Bondy's case, then the liver nuclei should be preferentially inhibited resulting in lower activities than brain nuclei; but this

presupposes that the observations by Dutton and Mahler of the differential stability of the polymerase A activity of brain and liver nuclei is also valid for their polymerase B activity--and this would not account for an increase in polymerase B activity in brain during maturation. In any case, the resolution of this apparent discrepancy must await more comprehensive data on the enzyme systems responsible for RNA metabolism, particularly the rate of degradation of nuclear RNA in brain as a function of age.

All the aforementioned data consistently agrees that by age 18 days when net increase in organ content of DNA and cellular content of RNA has ceased,[22,23,24] cells of the cerebral cortex have at least a 2-fold higher rate of RNA synthesis (6-fold higher with tissue slices) than cells of the cerebellum and most other regions of the brain (Bondy and Waelsch's data[50] suggest that caudate and hippocampus have levels of RNA polymerase B- catalyzed RNA synthesis activity comparable to that of cortex), and hence presumably a proportionally higher rate of RNA turnover. The functional significance of this regional difference in RNA metabolism measured *in vitro* is not known. However, the data of Giuffrida *et al.*[51] indicate that the difference is predominantly due to a 2-fold higher rate of RNA polymerase B- catalyzed RNA synthesis in the cerebral cortex since the level of RNA polymerase A-

catalyzed RNA synthesis is nearly equal in cerebral cortex, cerebellum and brainstem. Since RNA polymerase B is thought[58] to catalyze the synthesis of messenger-like RNA while RNA polymerase A synthesizes rRNA, this suggests a higher rate of synthesis and utilization of mRNA in the cells of the adult cerebral cortex (and possibly caudate and hippocampus) in comparison to other brain regions.

It is not known to what extent the high RNA polymerase B activity of adult cerebral cortex is due to the higher proportion of neuronal nuclei (25%) in this region of the brain or whther it is due to a high RNA synthesis activity specific to the neurons and/or non-neuronal cell types of the neocortex.

Guiffrida et al.[51] have demonstrated that within a given region of the brain different classes of nuclei are found having differences of at least 2-fold in their RNA polymerase activities. Neuronal nuclei were found to be more active than oligodendroglial or astrocytic nuclei.[51,59,60]

Yamagami et al.[35] have studied the composition of rat brain nuclear RNA at various stages of development and found that as the brain matures the high molecular weight components decrease. Their results indicate that during the maturation of the brain, both mRNA and rRNA are decreased

significantly in the nuclei after the thirtieth day and, subsequently, this decrease continues until the nuclear RNA is predominantly composed of small molecular weight RNA molecules in the adult brain.[74] No evidence has yet appeared indicating to what extent these results represent a preferential decrease in the synthesis of high molecular weight RNA species[42] and/or to increased nuclear RNA degradation[61] in the adult.

These developmental changes in cellular RNA correspond to a decline in the rate of protein synthesis[41] and would be expected to be accompanied by a qualitative shift in the mRNA species as the genetic program synthesizing mRNA coding for proteins required for cell division switches sequentially to the synthesis of mRNA species coding for the synthesis of proteins required for cell differentiation, cell growth and finally, maintenance functions of the mature cell. Thus at early stages of development brain RNA characteristics are compatible with the rapid rate of protein synthesis observed. There is a higher requirement for rRNA in the rapidly dividing cells.

1.12 Changes in brain RNA content and composition during aging

Atrophy and weight loss of the brain are the most common features of aging. This is accompanied by morphological changes in neuronal perikarya, neuropil, glial elements and

blood vessels which are believed to be responsible for the concurrent deterioration of function.[62] It is not known to what extent the changes in morphology of aging brain represent genetically coded, time-dependent changes in the cell or result from exposure to harmful environment. Such factors as errors introduced into protein synthesis, chronic ischemia, slow virus infection, nutritional deficiencies, intoxications, and failure of auto-oxidation are among those to be seriously considered. Little is known about the molecular processes underlying "normal" cell senescence. It is hoped that information as to the content, composition, synthesis and turnover of RNA in brain during aging will yield insight into the molecular events responsible for or contributing to the complex processes of cell degeneration and cell death.

According to Hollander and Barrows[63] whole brain RNA/DNA ratio in the C57 BL/6J mouse and Wistar rat does not change during aging. Chaconas and Finch[64] found a slight decrease in the RNA/DNA ratio of the striatum, but little or no decline in other regions of the C57 BL/6J mouse brain. Hyden[65] studying individual anterior horn spinal neurons of humans found a progressive decline in the total RNA content of these cells after sixty years. Ringborg[66] has also reported a similar decline in the total RNA content of single pyramidal cells of rat hippocampus. In the latter, the amount of RNA per cell increases from 24 pg in newborn rats to 110 pg

for mature rats and, subsequently, drops to 53 pg in very old rats. In the very old rats, the G+C/A+U ratio of the RNA of these cells rises from 1.66 in mature rats to 1.95. Thus, individual neurons in the CA_3 layer of the hippocampus of 36-month old rats contain about half the RNA found in these cells in 2-month old rats. Wulff and Freshman[67] using a microspectrophotometric technique for quantitating the total RNA content of single cells found a slight RNA loss in spinal motoneurons and cerebellar Purkinje cells of aged rats, but found no change in the RNA content of neurons of the supraoptic nucleus of the hypothalmus.[68]

It is, perhaps, significant that increases in cellular RNA content have not been reported to occur with aging. The relationship of those decreases in cellular RNA which have been demonstrated to the concomitant development of deficits in integrated brain function and behavior remains to be elucidated.

It must be noted that upon such basal life-cycle changes in cellular RNA content are superimposed short-lasting, reversible fluctuations in RNA content that result from increases or decreases in functional demand.

1.2 Turnover of Brain RNA

In adult rat, the half-life of both the RNA and proteins in brain ribosomes was found to be 12 to 18 days.[69,70] In 35-day old rat, brain rRNA has a half-life of 6 to 7 days.[70,71] Both the RNA and protein components of ribosomes within each age group turn over synchronously.[72] This age-dependent decrease in the turnover of ribosomes appears to be peculiar to the brain, since in a comparative study of other rat tissues Menzies et al.[73] found no age-dependent differences in the turnover of rRNA in liver, spleen, lung or intestinal mucosa. The half-life of adult rat liver rRNA has been found to be about 5 to 7 days.[74,75] These figures indicate a significant tissue-specific difference in the turnover rates of rRNA in the liver and brain of adult rats. In the adult animals brain rRNA turns over at about half the rate of liver rRNA. Since rRNA constitutes nearly 90% of the total cellular RNA[76] its turnover rate would be expected to have a decisive bearing on such processes as chromatolysis following axonal section.[97] A half-life of at least 6 days is compatible with estimates of histological methods of the rate of chromatolysis.[77] However, the disappearance of Nissl staining after severe anoxia and possibly also after intense chronic stimulation and stress,[104-109] is too rapid to be accounted for by failure of RNA synthesis in conjunction

with a normal rate of rRNA turnover. Rapid disappearance of Nissl substance may hence involve activation of ribonucleases present in the cytosol or associated with ribosomes.

No age-associated difference has been observed in the turnover of tRNA. This class of RNA exhibits a double exponential turnover pattern; one component has a half-life of 5 to 8 days, similar to that of liver tRNA[74,75] and the second component 13 to 16 days.[70,72,78] It is not known to what extent these results reflect differential turnover of different components of tRNA population within a single cell type or to differential turnover of the total tRNA population within different cell types such as neurons and glia.

Mitochondrial RNA of rat brain has been shown to have a half-life of 11.6 and 10 days in young and adult animals respectively.[70]

Data on the turnover rates of mRNA in rat brain indicates the presence of at least two populations of mRNA. A more labile fraction representing most of the mRNA turns over with a half-life of less than 4 hours,[79-83] and another smaller population of mRNA molecules turns over with a mean half-life of 10 to 12 hours.[84] Reports that polysomes from rat cerebral cortex[43] and whole brain[41] decrease in stability during maturation presumably due to an enhanced susceptibility to degradation of the mRNA in the polysomes would suggest a higher rate of mRNA turnover in the adult; however, direct

data on developmental changes in the turnover rates of mRNA is lacking.

There is little available data on RNA turnover in the nuclei of brain cells. Bondy[78] studied the turnover of *in vivo* labelled nuclear RNA in whole brain of adult rats and found that all of the acid-insoluble radioactive label lost from the nucleus in the first 18 h after intracranial injection of ^{14}C-cytidine could be accounted for by the increased labelling of cytoplasmic RNA. That is, the decrease in specific radioactivity of newly synthesized nuclear RNA was due to its rapid conversion to cytoplasmic RNA and transport out of the nucleus. Within the nucleus of adult brain cells there would thus appear to be little degradation of RNA to acid-soluble products of the type reported for HeLa cell nuclei by Harris.[85,86] Nuclear RNA exhibited a heterogeneous breakdown pattern with half-lives of from 26 h to 11.5 days. The specific radioactivity of nuclear RNA did not fall below that of the cytoplasmic RNA fractions in the manner which one would expect if a simple precursor-product relation existed between nuclear and cytoplasmic RNA. This suggests that a large amount of the RNA synthesized in the nucleus is not quantitatively transferred to the cytoplasm but remains in the nucleus. The extent to which nucleotides released from heterogeneous nuclear RNA (HnRNA) during its degradation are

capable of competing with the free nucleotide pool and thus of being re-utilized in RNA synthesis is not known. Watts[61] has reported that the turnover rate of nuclear RNA is more rapid in the adult than in the infant brain. The combined evidence suggests that in the adult, newly synthesized RNA is processed and degraded in the nucleus as well as transported to the cytoplasm at an enhanced rate.

It should be emphasized that the available information on RNA turnover in brain has been derived from a complex mixture of cell-types which may differ considerably in their RNA content and metabolism.[59,60,86-89] Watson[90] has reported that RNA turns over more rapidly in neurons than in glia and neuronal RNA turnover is markedly subject to variation depending on the functional activity of neurons, the physiological condition of the organism and its environmental status. Information as to the turnover rates of different classes of RNA (and of particular molecular species within particular class) within a single cell-type under various physiological conditions and states of neural activity is not yet available.

1.3 Changes in Brain RNA Content and Composition Accompanying Sensory Stimulation, Physiological Challenges and Learning Experience

Consistent with the brain's specialization for detecting and responding to changes in its ambient stimulus field there have been numerous reports[65,91-97] of

perturbations in the steady state content and composition of RNA of adult brain. Such changes have been demonstrated to occur as a consequence of ,or concomitant with ,changes in the functional state of the whole brain, specific brain regions, subregions, or the activity of specific neurons.

When adult rats were kept first in the dark for 3 days and then exposed to the light and sounds of a laboratory for 15 minutes, the polysome to monosome ratio increased by 83% in the cerebral cortex but was not affected in the liver.[98] Evidence has also been reported indicating that the total RNA of neurons in the auditory cerebral cortex is markedly elevated within 1 hour of a 60-minute exposure of rats to moderately intense white noise. The RNA content of these neurons returns to control baseline levels within 24 hours.[99]

On the other hand, convulsions produced by methionine sulphoximine or electroshock caused a disaggregation of polysomes.[100,101] A more detailed study[102] of the time-course of electroshock showed a decrease in number of polyribosomes during the first 15 minutes, and an increase over the following 15 to 30 minutes. By 60 minutes after electroshock no significant difference from control was observed.[103]

Other workers[104-109] have reported a rapid turnover of ribonucleoprotein (Nissl substance) and a decrease in total cellular RNA content under conditions of chronic

stimulation and stress. On the basis of such observations, Hyden has proposed that moderate neuronal excitation produces increases in RNA as an adaptation to increased functional demand on the neurons whereas intense chronic stimulation produces a decrease in neuronal RNA content through (1) slowing down RNA synthesis, possibly by diversion of ATP to other metabolic needs of the cell, and/or (2) increasing the rate of RNA degradation.

Mild sensory stimulation of the kind which increases neuronal RNA content appears to result from a non-specific increase of all classes of cellular RNA.[52] That is, the increased RNA content is not due to a preferential or disproportional increase in the quantity of a particular class of RNA molecules. Hyden[110] has performed base composition analyses of the increased neuronal RNA content elicited in response to increased physiological stimulation of neuronal activity and found that the new RNA has the same base-ratio characteristics as the bulk of RNA present in control cells. Whether the RNA which remains after intense chronic stimulation exhibits a base composition different from that of resting cells has not been determined. Shashoua has reported that KCl-induced convulsions and generally stressful physiological conditions resulted in no detectable base composition changes in the RNA of whole goldfish brain.[145] However, the occurrence of local changes in specific brain regions would not be detected in such an analysis of whole brain RNA.

Although changes in RNA content serve as a reliable indicator of alterations in the metabolic and functional state of neurons, a deeper understanding of the significance of these changes will require direct electrophysiological monitoring of the bioelectric activity of neurons as well as a more careful examination of the specific contribution of such parameters as stimulus frequency, intensity and duration to the net effects on cellular RNA content, composition and metabolism.

Since m-, t-, and r-RNA subserve protein synthesis it has been anticipated that under those conditions in which neuronal RNA content (or rate of synthesis) is increased, there might be a comparable, even greater concomitant effect on protein content and synthesis. Sufficient comparative data has not accumulated to test this hypothesis.[9,111-113]

Numerous reports have appeared demonstrating increases in the total content[114-117] as well as changes in the base composition[114,118-124] of neuronal RNA during the acquisition of a new behavior pattern.[125-131] Trained mice, compared to quiet and yoked mice, showed greater incorporation of radioactive uridine into brain[117,132] nuclear and ribosomal RNA, greater incorporation of uridine into polysomes isolated from brain,[113] and a higher ratio of brain polysomes to free ribosomes.[132] These results have been confirmed by autoradiographic studies with mice[134] and rats given similar

training.[135,136] These changes were not observed in liver and kidney.[117,132-133]

Reports[117,125] of increased uridine incorporation into RNA during training experience cannot always be reliably interpreted as indicating an actual increase in the rate of RNA synthesis since increased labelling could also result from a reduction in the size of the endogenous unlabelled (most likely intranuclear) ribonucleotide precursor pool.[130,137] Changes in ribonucleotide precursor pool sizes have, in fact, been observed under such conditions.[75] Increased incorporation of label into RNA might also be observed if a decreased rate of RNA degradation were combined with an unchanged rate of RNA synthesis. However, results obtained following brief pulse times are measures of the initial rate of synthesis and are not significantly influenced by the rate of RNA degradation.[57] Measurements of the turnover rate of neuronal RNA under these conditions have not been reported.

The increases in neuronal RNA content which have been detected in learning experiments may be an unspecific sign of increased activity, not related to a specific learning or information consolidation process. In view of the large influence of the organism's behavioral history upon these measures,[112,139-140] it would seem to be necessary to use as controls animals who have been previously adapted to some of the simple stimulus components of the training situation in

order to identify changes in molecular species specific to the occurrence of instrumental learning during training experience.

It has been reported[114,118-121] that in contrast to conditions of increased physiological sensory and/or motor activity in non-learning situations, the RNA content increases which accompany learning exhibit base-ratio changes in the direction of the base composition characteristics of mRNA (i.e., higher adenine and uracil content). This would indicate that individual neurons are capable of discriminating between sensory information processing contingent upon the acquisition of a new behavior and activation which does not entail the acquisition of new information. Such specificity of neurochemical response indicates that the regulation of neuronal RNA metabolism and possibly gene expression by sensory information is mediated via the receptive pole of the neuron at which the electrically-coded information is transduced into molecular messengers. RNA metabolism is not directly dependent upon or coupled to the bioelectric response output or electrophysiological activity of neurons,[138,143,154] but would seem rather to be regulated by neurohormones presumably acting upon receptors of the post-synaptic membrane. The nature of the intracellular molecular messages into which sensory information is transduced and the intracellular causal sequence of events by which the modulation of RNA metabolism

is effected remains to be elucidated. Also, it is not yet possible to infer from the experimental evidence available that new molecular species of RNA are synthesized through the release of genome information in order to account for the observed base-composition changes in neuronal RNA. Although hormonal induction of genome information has been well documented in other biological systems, alternative explanations of the reported alterations in RNA base-composition in neurons have not been adequately tested. Such changes might be due to (1) a change in the quantity of one or more species of mRNA or a differential quantitative shift in the rate of synthesis or degradation of two classes of RNA resulting, for example, in a net increase in mRNA/rRNA ratio, (2) enhanced activity of terminal addition enzymes; for example, of the kind catalyzing the post-transcriptional adenylation of the 3'-terminal end of mRNA, or (3) intercellular transfer of specific RNA molecules between glia and neurons.[91,110] Direct evidence of the occurrence of qualitatively new molecular species of RNA or protein during learning (which might be obtained by competitive hybridization[141,142] or electrophoretic profile studies) is not yet available to supplement the base-composition data and facilitate the interpretation of their significance to the molecular mechanisms underlying learning and memory formation.

The questions of whether the changes in neuronal RNA base-composition which are correlated with the acquisition and consolidation of sensory information represent the synthesis of qualitatively new species of mRNA and whether eventually new species of proteins[12,114,144] also are formed during the establishment of a new behavioral response have not been definitively answered.

However, sufficient data have, nevertheless, accumulated to implicate at least an indirect involvement of RNA in the molecular processes which underlie changes in neuronal function brought about by changes in sensory stimulation and learning experience. The determination of how transient perturbations of the steady-state neuronal levels of RNA are effected will require a more detailed understanding of the enzyme systems responsible for the synthesis and degradation of RNA and the mechanisms by which they are regulated, since it is through these mechanisms that changes in neuronal RNA are most likely coupled to neuronal membrane receptors.

The particular concern of the present study is the RNA degradation system of the cell, its participation in cellular RNA metabolism and its contribution to the changes in cellular RNA which have been observed to occur in the course of brain development, aging and during alterations in neuronal function in the adult brain.

1.4 The Role of the RNase Enzyme System in RNA Metabolism

It is probable that the determination of the lifetime of various RNA molecules by RNases operates as a key factor in the control of cellular biosyntheses. Considerable effort has, therefore, been invested in studies of the functional aspects of the class of enzymes which have in common the ability to split phosphodiester bonds in RNA. The participation of enzymes cleaving the diester linkages between ribonucleotides has been implicated in diverse aspects of cellular metabolism. Biological functions for which some evidence is available include (1) the maturational processing of precursor transcription products, and (2) the degradation of endogenous ribosomal, messenger, transfer and nuclear RNA. Other possible functions for which experimental evidence is more tenuous include (3) defense against foreign RNA , and (4) provision of ribonucleotides for reutilization by the RNA synthesizing apparatus.

There is sufficient evidence now to conclude that in both procaryotic and eucaryotic cells mRNA[146-150], rRNA[151-153,155,156] and tRNA[157,158] are all transcribed as precursor RNA molecules which are subsequently trimmed of their extra nucleotide sequences by maturation endoribonucleases to form functionally mature RNA molecules. The processing of the large nuclear pool of rapidly turning over

HnRNA appears likely to involve (1) site-specific endoribonucleases which produce selective cleavage(s) of the primary transcription products, and (2) RNases of more general specificity which degrade the non-functional cleavage products to monoribonucleotides. Considerable research is currently being directed toward elucidating this critical role of RNases in nuclear RNA metabolism and in the provision of functionally mature RNA molecules to the protein-synthesizing apparatus of the cytoplasm.

The RNase enzyme system also regulates the functional levels of both nuclear and cytoplasmic RNA content by its participation in RNA degradation. The determination of the lifetime of various RNA molecules by RNases is of obvious importance in regulating the availability and limiting the extent of use of functionally mature RNA molecules in protein synthesis.

A third possible function of RNases suggested by Herriot[159] is the degradation of infectious viral RNA. That a barrier exists to infection of plant and animal cells by naked viral RNA is well established,[160,161] and intracellular RNase activity is a likely candidate for this function.[162,163] However, in plants the overall content of RNase in leaves is not correlated with susceptibility to infection by intact virus.[164] Direct evidence linking RNases

with protection against infection by RNA virions in plant, animal or bacterial cells has not yet been reported.

Finally, a possible function of RNA depolymerizing enzymes which has received little consideration is the provision of ribonucleotide precursors to the RNA synthesizing apparatus of the nucleus. Some question exists[165] as to the sufficiency of nuclear de novo nucleotide synthesis and nucleotide influx from the cytoplasm as mechanisms for the replenishment of ribonucleoside triphosphates within nuclei during RNA synthesis. It has been alternatively hypothesized[165,166] that rate-limiting control of RNA synthesis may be achieved through the regulation of the free ribonucleotide pool size by the rate at which ribonucleoside-5'-phosphates or ribonucleoside-5'-diphosphates are liberated from nuclear polyribonucleotide storage molecules by the action of exoribonucleases within the nucleus. Such a mechanism would explain (1) how the processes of RNA synthesis and RNA degradation are tightly coupled or coordinated to achieve steady-state turnover rates, (2) how RNA synthesis can proceed at rapid rates without rapid depletion of the small pools of free ribonucleotides in the nucleus,[165] and (3) a possible function of the rapidly turning over homopolyribonucleotides[167-170] and other nuclear RNA which does not reach the cytoplasm and whose

function in the nucleus is to date undetermined.[171-175] Ribonucleoside-5'-phosphate-forming ribonuclease activities capable of fulfilling such a putative function have been identified in guinea pig liver,[176,177] rat liver[178] and Ehrlich ascite carcinoma[179] cell nuclei.

The ribonuclease enzyme system thus modulates the levels of functional RNA molecules in the cell by participating in the biosynthesis and degradation of functionally mature RNA. Much remains to be learned as to the specific contribution of this group of enzymes and its individual components to the specific changes in cellular metabolism accompanying each stage of the cell cycle (cell division, cell differentiation, cell growth, cell maintenance and cell degeneration).

1.5 Regulation of RNase Activity and RNA Degradation

It must be postulated out of logical necessity that all tissues and cells possess an enzyme system for degrading RNA molecules. If this enzymatic apparatus were allowed to function uncontrolled it would completely hydrolyze the cellular RNA. Since this does not occur, it follows that regulatory mechanisms must exist which control the functional levels of RNase activity and prevent the indiscriminate breakdown of RNA within the cell.

The molecular mechanisms by which intracellular RNase activity and RNA degradation are regulated are little understood. The question as to which or whether a particular type of RNase in a cell specializes in attacking only one class of RNA has not been definitively answered. While all classes of RNA are likely to be competent substrates for any one RNase in variously manipulated *in vitro* conditions, the intracellular situation of the RNA and of the enzyme may impose considerable restriction *in vivo*. Thus, apart from the degree of specificity inherent in the enzyme-substrate interaction which may entail recognition of primary, secondary and tertiary structural features of the substrate, additional constraints on substrate-enzyme interation may depend on the functional level of various RNases as well as their accessibility to substrate. Several means by which such regulation or restriction of enzyme-substrate interaction may be achieved *in vivo* include: (1) spatial segregation of substrate and enzyme by differential intracellular compartmentalization; (2) complexing of RNA with protein to form ribonucleoprotein structures resistant to the action of RNases; (3) complexing of enzymes with inhibitor molecules; (4) allosteric modulation of the substrate-binding or catalytic activity of RNases by small effector molecules, and (5) alterations in the rate of *de novo* synthesis or turnover of RNases.

Spatial segregation of the enzyme is clearly involved in the prevention of indiscriminate degradation of RNA by the large amounts of RNase present in lysosomes.[180] Lysosomal RNase is unlikely to be involved in the turnover of a single specific class of RNA. The activity of lysosomal RNase depends on the fusion of vacuolized RNA particles with the lysosomes or the rlease of lysosomal RNases into the cytosol following a change in lysosomal membrane permeability. Such lysosomal membrane changes have been reported to occur under conditions of stress[180,181] and are followed by a considerable breakdown of messenger and ribosomal RNA.

Upon being synthesized most RNA becomes associated with specific proteins. The protein-associated state of the RNA provides an obvious means of regulating its accessibility and susceptibility to degradative enzymes. That RNA-associated proteins restrict the activity of RNases <u>in vivo</u> is indicated by the fact that in intact polysomes, rRNA is unaffected by levels of RNase which readily attack the inter-ribosomal segments of mRNA and the rRNA of free ribosomal subunits.[80,182] Ribonucleoprotein particles may even bind RNase without destruction of their RNA.[183,184] Bovine pancreatic RNase A-catalyzed degradation of ribosomes has been demonstrated to depend on their functional state, and Yanofsky[354] has suggested that mRNA while associated with ribosomes is afforded some protection from the action of RNases.

Associated proteins thus confer resistance upon the RNA of ribonucleoprotein complexes by imposing constraints on the steric accessibility to RNases of specific sequences in the RNA molecules. Maturational processing and turnover of RNA must, therefore, proceed in a regulated sequence of steps with exposed cleavage sites being attacked first possibly by site-specific endoribonucleases. Initial cleavages would be expected to induce conformational changes in the ribonucleoprotein structure possibly accompanied by the dissociation or loosening of bound protein and hence the exposure of new cleavage sites which could then be attacked by RNases of more general specificity. Thus, the degradation of a single RNA molecule might entail the participation of several different RNases at different stages of its degradation. Also, the concerted action of RNases and proteases in the degradation of ribonucleoprotein is suggested by the synchronous turnover of the RNA and protein components of ribosomes.[69,70] Any impairment in the fidelity of transcription would result in defective RNA molecules which could not participate in ribosome and informosome assembly. This defective RNA unprotected by function would thus be more susceptible to attack by RNases and, therefore, would be degraded rapidly, whereas the functional pool of RNA would undergo normal turnover. Such a mechanism of differential degradation of functional versus non-functional RNA molecules

would minimize the manifestation of transcriptional errors at the level of protein and cell function. Thus, the control of RNA turnover could depend greatly on changes in local ion concentrations and other factors which dissociate ribonucleoprotein complexes as well as upon more direct regulation of the functional levels and activities of specific RNases.

The complexing of enzymes with specific protein inhibitors has been demonstrated in some cells as a mechanism for controlling the activity of such catabolic enzymes as proteases,[185] deoxyribonuclease[186] and pH 7.8 RNase.[187] The functional level of the latter enzyme thus depends on the partitioning between active and inhibitor-bound forms of the enzyme and could be regulated by any factors affecting this balance. The reported[188] absence of detectable RNase inhibitor protein in nuclei may partially account for the rapid turnover of RNA in the nucleus, whereas RNA transported out of the nucleus into the cytosol which contains a large excess of RNase inhibitor, escapes rapid degradation.

Some evidence does exist[189] for the control of functional levels of intracellular RNases through the regulation of the rate of synthesis and translation of the mRNA molecules coding for these enzymes. The factors responsible for effecting this control are unknown. The factors regulating the lifetime and turnover of specific RNases are also unknown.

Much remains to be understood as to the mechanisms by which RNA degradation is regulated and coordinated with RNA synthesis.

1.6 Intracellular RNases of Non-Neural Mammalian Tissues

Intracellular RNases of mammalian tissues have not been purified to homogeneity and knowledge of their structure and function does not approach in completeness that for bovine pancreatic RNase A or RNase T_1. Extensive study of this enzyme system in animal tissues has, nevertheless, revealed it to be complex, consisting of many distinguishable components widely divergent in their structural and functional properties. The intracellular RNase system of rat liver has been studied more extensively than that of any other tissue, and the following discussion will hence deal primarily with those components of the RNase system which have been identified and more or less thoroughly characterized in this tissue.

1.61 Acid RNase

An RNase with an acid pH optimum has been characterized and partially purified by a number of investigators.[190-193] This enzyme is highly unstable and is rapidly inactivated by heat and dilute acid. It has a molecular weight of about 24,000 to 28,000 Daltons,[194] a pH optimum

of 5.8, and no metal ion requirement. Acid RNase has been reported to degrade partially hydrolyzed RNA more rapidly than high molecular weight RNA,[192] to hydrolyze all purine and pyrimidine phosphodiester bonds (although it has a pyrimidine bond preference) to 3'-ribonucleotides with the intermediate formation of 2', 3'-cyclic ribonucleoside phosphates[191-195] and hence to leave no undialyzable RNA core.[196,197] The single-stranded form of RNA is specifically or preferentially attacked. Although a high purification of this enzyme from rat mammary carcinomas has recently been reported,[189] definitive substrate specificity studies with highly purified enzyme preparations have not yet been done.

Initial studies of the intracellular distribution of this enzyme found it to be associated primarily with the crude mitochondrial fraction.[198,199] De Duve et al.[200] subsequently demonstrated that this enzyme along with other acid hydrolases is associated not with mitochondria per se but with a separate class of organelles which they named "lysosomes." Reid and Nodes[196] obtained similar results and concluded that acid RNase was present in cell particulates intermediate in size between mitochondria and that class of lysosomes containing acid phosphatase. Rahman[201] also studied the intracellular distribution of acid RNase in comparison with that of several other acid hydrolases and

found it to be present in a class of lysosomes similar to that containing cathepsin D but distinguishable from those lysosomes containing acid phosphatase and cathepsin C. De Lamirande and Allard[202,203] also found that acid RNase is not contained in the same class of cell particulates as acid phosphatase, but concluded on the basis of the similarity of its distribution with glutamate dehydrogenase that it must be present predominantly in mitochondria. Considerable amount of the total cellular acid RNase activity has also been recovered in the high speed supernatant fraction of cell homogenates.[194,202] It is not clear whether this fraction of the enzyme activity actually exists <u>in situ</u> in the soluble phase of the cytoplasm or whether it represents enzyme released from lysosomes during the tissue homogenization and subcellular fractionating procedures. Some evidence has been obtained[194] suggesting that the acid RNase activity of the cytosol may be due to an enzyme different from that found in the crude mitochondrial fraction.

1.62 pH 9.5 RNase

An alkaline RNase with a pH optimum of 9.5 was first detected by Rahman[204,205] in rat liver. It is strongly inhibited by most monovalent and divalent cations and this inhibitory effect is not due to ionic strength. However, at a concentration of 0.5 to 1.0 x 10^{-3} M Mg^{++}, unlike other divalent cations, produces a 30 to 40% activation of this

enzyme. Inhibition by EDTA can also be overcome by Mg++ at a suitable concentration. This enzyme is heat and acid labile and exhibits substrate inhibition with an RNA concentration of more than 2.0 mg/ml. The above characteristics have been reported[204] for the unpurified enzyme and no purification procedure or substrate-specificity data have yet been published. In a study of its intracellular distribution Rahman[206] reported the pH 9.5 RNase to be associated primarily with mitochondria and microsomes. Little of the total cellular content of this enzyme was recovered in the lysosomal and supernatant fractions, although lysosomes possessed the highest specific pH 9.5 RNase activity.

1.63 pH 7.8 RNase

An alkaline RNase with a pH optimum of 7.8 has been extensivley studied and partially purified.[191-193,199,207] This enzyme exhibits a broad activity maxima between pH 6.7 and 8.5,[208] it has no divalent cation requirement, and is stable at 70° for 5 minutes. It hydrolyzes RNA more rapidly than poly U and poly C and shows no activity toward poly A and poly G. It appears to split principally pyrimidine phosphodiester bonds to yield ribonucleoside-3'-phosphates via 2', 3'-cyclic pyrimidine nucleotide intermediates, and leaves a resistant undialyzable oligoribonucleotide core.[196,208] This enzyme is thus similar in its functional properties to bovine pancreatic RNase A. pH 7.8 RNase has been reported

to have a molecular weight of between 11,500 to 12,000 Daltons.[194] Beard and Razzell[208] have achieved a 3,000-fold purification of this enzyme from hog liver and Gordon[209] has purified rat liver pH 7.8 RNase 6,000-fold.

Results obtained by various workers concerning the intracellular distribution of this enzyme are not entirely in agreement. Roth[207] separated the rat liver homogenate into only three fractions--nuclei, crude mitochondria, and supernatant--and reported that the total pH 7.8 RNase activity was recovered in the mitochondrial and supernatant fractions with a small amount of enzyme in the nuclei. Reid and Nodes[196] achieved a further fractionation of the crude mitochondrial fraction into mitochondria and lysosomes and reported that the highest specific activity of pH 7.8 RNase occurred not in mitochondria but in a class of lysosomes "less readily sedimented than those particles containing acid phosphatase." However, these investigators did not present their data in terms of the per cent distribution of the total activity. Rahman[206] subsequently confirmed that the lysosomal fraction possessed the highest specific pH 7.8 RNase activity (3-fold that of any other fraction) and found that the per cent recovery of total activity was lowest in the lysosomal and highest for the mitochondrial fraction. Fifty per cent of the total recovered pH 7.8 RNase activity was also approximately equally distributed between the

microsomal and supernatant fractions. De Lamirande and Allard[199] under their subcellular fractionation and assay conditions found pH 7.8 RNase to be evenly distributed among mitochondria, lysosomes and microsomes. Discrepancies in the intracellular distribution data probably reflect differences in assay procedures employed in different laboratories.

1.64 RNase Inhibitor

Pirotte and Desreux[210] first described an RNase inhibitor protein in the cytosol of guinea pig liver. The properties of the inhibitor from rat liver have been studied,[187,211,212,213,155,158] and it has been partially purified.[214] It has also been studied in mouse tissues,[215] mouse ascites tumours,[216] rat adipose tissue[217] and blood.[218] The rat liver RNase inhibitor is a labile protein readily inactivated by acid, heat, heavy metal ions such as Mg++ and Pb++ and by sulfhydryl-blocking reagents such as p-chloromercuribenzene sulfonic acid.[187,211]

The free inhibitor is present in large excess in the rat liver supernatant fraction as indicated by the inhibition by this fraction of sizeable quantities of added bovine pancreatic RNase A or free pH 7.8 RNase. There is also in most mammalian tissues a considerable quantity of inhibitor present which is complexed to the endogenous pH 7.8

RNase.[187,211] On treatment of the supernatant fraction with sulfhydryl-blocking reagents, acid, or heat the inhibitor is inactivated and the inhibitor-bound latent pH 7.8 RNase is released in active form.[211]

Some characteristics of the interaction between RNase inhibitor and modified derivatives of bovine pancreatic RNase A have been studied.[219] It was determined that the reaction between the two proteins is not dependent on the active site of the RNase, nor on the free amino groups of the enzyme. Interaction is, however, strongly dependent on a relatively native configuration of the RNase and on hydrogen bonding. There is considerable evidence that free sulfhydryl groups are essential for inhibitor activity. Roth[211] has suggested that since pancreatic RNase A has no free sulfhydryl groups, the formation of a disulphide bond between RNase and inhibitor is unlikely. Rather, the sulfhydryl group(s) of the inhibitor may participate in the formation of hydrogen bond(s) with the enzyme, or may be required to maintain the active conformation of the inhibitor.

RNase inhibitor is active principally in the pH range between 7 and 9 and has been reported to produce little or no inhibition of acid RNase[195,213] or pH 9.5 RNase.[204] The specificity of the inhibitor action is not wholly accountable in terms of the pH dependence of the inhibitor-enzyme interaction since the inhibitor produces significant inhibition of

bovine pancreatic RNase A assayed at pH 5.8,[187,213] whereas RNase T_1 which is similar in several respects to pancreatic RNase A was not inhibited when assayed at pH 7.8.[213]

RNase inhibitors isolated from different mammalian sources are not specific and do cross-react in varying degrees with heterologous pH 7.8 RNases.[212]

There are considerable tissue- and species-specific differences in the relative amounts of free and RNase-bound inhibitor.[187]

RNase inhibitor has recently been purified to near homogeneity[220,221] and it has been found to be an acidic protein with a molecular weight of 50,000 to 60,000 Daltons.[222]

1.65 Ribosomal RNase

Another alkaline RNase has been detected in ribosome and polysome preparations from rat liver.[223-225] This ribosomal RNase has a pH optimum of 8.5, is relatively stable in acid, but rapidly inactivated above 55°. It is stimulated by an optimal divalent cation (Ca++ or Mg++) concentration of 10 mM and a monovalent cation (K+ or Na+) concentration of 50 mM. Its molecular weight has been estimated to be 37,000 Daltons. Unlike the pH 7.8 RNase of the cell sap, and mitochondrial fractions, ribosomal RNase does not degrade poly U. Like the pH 7.8 RNase of the cell sap it exists in a latent form bound to RNase inhibitor. Although

direct inhibition of the purified enzyme by the cytosol or purified inhibitor has not yet been demonstrated, several workers have reported that polysomes are stabilized by RNase inhibitor preparations.

The fact that, despite considerable effort, it has not been possible to isolate polysomes free of RNase activity suggests that the bound RNase may be an integral part of polysome structure and may be involved in their normal function. Roth[226] has made the interesting observation that the activity of the alkaline RNase present in rat liver microsomes must be functionally intact for the incorporation of ^{14}C-1-leucine into protein. This suggests that this enzyme may play a critical role in the regulation of protein synthesis.

1.66 Nuclear RNases

Many investigators have attempted to detect the enzymes responsible for the rapid turnover of RNA in the nucleus since the activity of these enzymes may regulate the kind and amount of RNA that does find its way into the cytoplasm.

Heppel et al.[227,228] have detected an alkaline RNase with a pH optimum between 8.5 and 9.5 in nuclei of guinea pig liver. This enzyme is inactivated below pH 6 and above 50°. It hydrolyzes poly A and poly U to ribonucleoside-5'-phosphates

and oligoribonucleotides of two to six units in length terminated by 5'-phosphates.[176] An 80-fold purification of this enzyme has been achieved by Razzell.[229]

Cunningham and Steiner[230] have reported further properties of what appears to be the same enzyme.

A polyribonucleotide phosphorylase has also been reported to be present in guinea pig liver nuclei[177] and HeLa cell nuclei.[231] This enzyme is supposedly associated with chromosomes and may participate in the breakdown of newly synthesized heterogeneous nuclear RNA (HnRNA) to ribonucleoside-5'-diphosphates.

An RNase activity has also been detected in the nucleolar fraction obtained from normal,[232,233] neoplastic[233,234] and regenerating[235] rat liver as well as from HeLa[236,237] and L cells.[238] This enzyme is an endoribonuclease and has been implicated in the processing of 45 S precursor ribosomal RNA into mature 18 S and 28 S rRNA. However, this putative function remains to be definitively demonstrated.

Other endoribonucleases analogous and possibly homologous to those functioning in the maturational processing of precursor tRNA[239,240] and precursor rRNA[241-243] in bacteria have not yet been demonstrated in mammalian cells.

Stein and Hausen[244,245] have reported an interesting enzyme (RNase H or hybridase) from calf thymus which

specifically degrades the RNA moiety of DNA-RNA hybrids. The recent finding that RNase H activity appears to be a universal property of reverse transcriptases[246,247] from RNA tumor viruses has led to the suggestion that the biological role of this RNase activity is to remove the oligoribonucleotide which normally functions as a primer in DNA replication[248,249] and which becomes covalently linked[250,251] to the newly synthesized DNA chain. Stimulation of *in vitro* transcription by rat liver RNA polymerase B of native rat liver DNA and chromatin has also been reported[252] to occur in the presence of rat liver RNase H. This stimulation was attributed to the supposed participation of this RNase in the release of newly synthesized RNA from the template.[252]

In general, studies of nuclear RNases have not achieved a high degree of resolution. Those enzymes which have been detected have not been extensively purified or characterized. Further work is required to define the role of these enzymes in the turnover of RNA in the nucleus and in the nuclear maturation of cytoplasmic RNA.

1.7 Changes in RNase Activities under Various Physiological and Pathological Conditions

A number of workers have monitored variations in the levels and intracellular distribution of various components of the RNase enzyme system in several tissues under various

physiological and pathological conditions with the expectation that such information might yield a better understanding of the functional role of these enzymes in the regulation of cellular RNA content and turnover.

Cellular RNase activity has been found to be elevated under several circumstances in which the RNA/DNA ratio is reduced[253-255] and protein synthesis[189,253-267] is reduced. Thus, decreases in RNase inhibitor or increases in free pH 7.8 RNase activity levels have been observed in tissues exhibiting a low rate of protein synthesis or an increase in their catabolic activities.[257-267] In some cases[189,257,258,265] increased acid RNase activity has also been demonstrated under similar conditions. These observations suggest that the decreased protein synthesis under these conditions is due to elevated functional levels of various cellular RNase activities and hence enhanced degradation of cellular RNA.

Conversely, increased RNA/DNA ratios and increased protein synthesis have been demonstrated[268-275] to be positively correlated with decreased functional levels of several cellular RNase activities. Thus, increases in the ratio of RNase inhibitor/free pH 7.8 RNase activity have been observed in tissues characterized by a high rate of RNA synthesis and/or cell division[268,275] such as in regenerating rat liver[276,277] and liver of hypophysectomized rats,[255,278] nephrotic kidney[279,280] and during compensatory renal

hypertrophy following unilateral nephrectomy.[281] In the case of nephrotic kidney[280] decreases in pH 9.5 RNase and acid RNase were also found to be positively correlated with the elevated rate of protein synthesis. These observations have suggested that the increased protein synthesis in these conditions may result from the preservation of mRNA and possibly also tRNA and rRNA.

RNA turnover studies[282,283] have indicated that in these situations the changes in cellular RNA content are due predominantly to changes in the rate of RNA catabolism rather than in the rate of RNA synthesis. On the basis of these findings, Shortman[272] has hypothesized that the changes in cellular RNA content and cellular metabolism accompanying changes in physiological conditions are effected through RNases by influencing the rate of RNA degradation. This work thus points to a need for a thorough investigation of the contribution of the RNase enzyme system to the changes in cellular RNA of brain which have been reported to occur in response to changes in physiological stimulation and during learning and memory formation.

1.8 The RNase Enzyme System of Brain

Whereas some information exists concerning the enzymes involved in the synthesis of RNA in brain, comparatively little is known of the molecular processes by which cellular

RNA content is post-transcriptionally controlled. Information as to the enzymes participating in the maturational processing of precursor transcription products into functionally mature transfer, ribosomal, and messenger RNA as well as in the degradation of these molecules is not available in the same degree of detail for brain as for many other mammalian organs.

Roth[187] in a comparative study of various rat tissues found that RNase inhibitor activity was highest in brain and hence presumably has a particularly important role in regulating the metabolism of RNA in this tissue. Ellem and Colter,[215] however, in a similar study of mouse tissues found that mouse brain contained very low levels of both pH 7.8 RNase and RNase inhibitor activity. Guroff[284] in a developmental study of rat brain RNase activity assayed at pH 5 found that the level of RNase activity expressed at this pH was lower in older animals than in young animals. Suzuki and Takahashi[285] studied the regional distribution of RNase inhibitor in rabbit brain and found that those areas (cerebral cortex, cerebellum, hippocampus) rich in neurons corresponded to the areas having highest RNase inhibitor activity. The maximal variation in the level of RNase inhibitor activity between the different brain regions studied was not more than 30%. These investigators have also reported a developmental profile for RNase inhibitor activity in rat

cerebral cortex. This component of the RNase system exhibits a sharp peak between the 5th and 10th day after birth, falls to near neonatal levels by the 13th day, and subsequently remains relatively constant. Takahashi, Mase and Suzuki[221] have also recently reported a high purification of RNase inhibitor from pig cerebral cortex. Datta and co-workers[286] have detected two RNase activities with pH optima of 5.4 and 7.9 in ribosomes isolated from goat cerebral cortex. Detection of significant activity required incubation periods of 14 to 18 hours. Both activities were tightly bound to the ribosomes and could not be solubilized. These workers have suggested that these ribosomal RNase activities may be responsible for the chromatolytic changes which ribosomes undergo during neuronal stress. An RNase activity with a pH optimum of 7.6 has been detected[287] in the cerebrospinal fluid of humans. Although its source and function were not determined, it was observed that the spinal fluid of patients with damaged CNS tissue (cerebral infarction, neoplasm, and demyelination due to multiple sclerosis) exhibited elevated levels of this RNase activity.

In general, the available information on the RNase enzyme system of brain is fragmentary and unconfirmed.

1.9 The Present Investigation

Because the RNase enzyme system plays such an important role in regulating intracellular RNA content and hence in regulating the availability of functionally mature RNA molecules to the protein-synthesizing apparatus of the cell, it is possible that any distinctive characteristics of this enzyme system in brain may have a direct relationship to the specialized function of this tissue. Also inasmuch as changes in brain cellular RNA content have been demonstrated to occur in response to learning and sensory stimulation, the role of RNases may achieve special significance in brain in comparison with other tissues such as liver which have been more extensively studied. Thus, for example, the enhanced levels of neuronal RNA resulting from sensory experience may be predominantly due to decreased RNA degradation which, in turn, may be due to decreased functional levels of intracellular RNase activity.

The investigation reported upon in this thesis was hence aimed at obtaining more detailed and comprehensive information about the characteristics of the RNase enzyme system in rat brain and at achieving some understanding of the contribution of the individual components of this enzyme system to the regulation of RNA metabolism in this tissue.

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II. MATERIALS AND METHODS

2.0 Materials

2.01 Experimental animals

Rats of the Wistar strain were obtained from the Vivarium of the Department of Zoology, University of British Columbia.

2.02 Chemicals

Triton X100 was a product of Rohm and Haas. Ultrapure sucrose and ammonium sulphate were obtained from Swartz/Mann. Para-chloro-mercuribenzoicacid was a product of Nutritional Biochemicals Corporation. This reagent was dissolved in .02 M Tris-HCl buffer, pH 8.9. Dithiothreitol (Cleland's reagent) was a product of Calbiochem. Solutions of dithiothreitol were freshly prepared in distilled water immediately prior to use. Gelatin was a product of Eastman Organic Chemicals and was purified by the method of Shortman.[214] DEAE cellulose with an exchange capacity of 0.84 milliequivalent monovalent anion per gram was obtained from BioRad Laboratories and was purified by the method of Peterson and Sober.[288] All other common laboratory chemicals were "reagent grade" and were used without further purification.

Fleischmann's yeast s-RNA prepared by the method of Holley[289] was the product of Calbiochem. Solutions of this substrate prepared in distilled water could be stored at 4° without exhibiting significant increase in acid-soluble A260. Highly polymerized double-stranded calf-thymus DNA was a product of Calbiochem, and was dissolved in .01 M NaCl. Bis-(p-nitrophenyl) phosphate (Na) was a product of Sigma.

Crystalline bovine pancreatic RNase A (ca. 50 Kunitz units/mg) was a product of Nutritional Biochemicals Corporation. Bovine serum albumin was a product of Calbiochem.

2.1 Methods

2.11 Preparation of tissue homogenates and extracts

Wistar strain white rats of body weight 200-400 g were decapitated and the whole brain quickly removed from the cranium and placed in a glass petri dish kept on ice. The tissue was weighed, suspended in ice-cold 0.1% (v/v) Triton X100 or 0.32 M sucrose in the ratio of one gram wet weight tissue: 9 ml of homogenizing medium, and homogenized at 0° with nine to ten strokes in a glass homogenizer fitted with a teflon pestle (Arthur H. Thomas Co.; clearance, 0.13-0.18 mm).

Tissue used for purification work was processed as above with the exception of being homogenized at 0° in a stainless steel OmniMixer (Ivan Sorvall Inc.) for 30 seconds at speed control 5, 30 seconds pause, and 30 seconds at speed control 6.

The 10% (w/v) tissue homogenates were centrifuged at 34, 800 x g for 60 minutes at 0-4° and the supernatant decanted and referred to as the "extract."

2.12 Fractionation of isotonic sucrose homogenates by differential centrifugation

For subcellular distribution studies isotonic sucrose homogenates were fractionated by differential centrifugation at 0° in a type SS-34 rotar of a Sorvall RC2 centrifuge and a Spinco rotar #50 of a Beckman Model L ultracentrifuge. Isotonic sucrose homogenates prepared as described in section 2.11 were centrifuged at 800 x g for 10 minutes, the crude 800 x g pellet was resuspended in a volume of ice-cold 0.32 M sucrose equal to 1/5 the volume of the initial homogenate (resuspension was carried out in a glass homogenizer, the pestle being gently rotated by hand), and centrifuged again at 800 x g for 10 minutes to yield the final 800 x g nuclear pellet. Supernatants from the preceding two centrifugations were pooled and centrifuged at 8,000 x g for 20 minutes to yield the 8,000 x g mitochondrial pellet. The 8,000 x g

supernatant was then centrifuged at 105,000 x g for 60 minutes (Spinco rotar #50) to yield the 105,000 x g microsomal pellet and the 105,000 x g supernatant or cytosol fraction. The final particulate fractions were each suspended in and brought to a final volume with ice-cold 0.32 M sucrose equal to one-half the starting volume of the homogenate, and rehomogenized at 0° with five strokes in a glass homogenizer.

2.13 Purification procedure

2.131 Ammonium sulphate fractionation

Powdered ultrapure ammonium sulphate was sprinkled onto the surface of a magnetically stirred crude enzyme extract maintained at 0° in an ice-water bath. The rate of addition of ammonium sulphate was controlled so as not to exceed the rate at which the salt dissolved. The amount of ammonium sulphate added to give a desired saturation was calculated according to the formula of Noda and Kuby.[290] After 30 minutes of additional stirring, the precipitates were collected by centrifugation at 34,800 x g for 15 minutes, suspended in ice-cold .02 M Tris-HCl, pH 7.4 and dialyzed at 4° in the cold room against the same buffer. Nessler's reagent was used to test for complete removal of $(NH_4)_2SO_4$ from the dialysates according to Umbreit, Brunis and Stauffer.[236a]

2.132 DEAE cellulose column chromatography of ammonium sulphate fractions

Diethylaminoethylcellulose was treated according to the procedure of Peterson and Sober.[288] The washed DEAE cellulose, suspended in .02 M Tris-HCl, pH 7.4 equilibrating buffer, was poured into columns at 4° in the cold room and allowed to settle under gravity. The packed columns were washed with 500 ml equilibrating buffer prior to being loaded with the dialyzed ammonium sulphate fractions. After the protein solution had completely entered the column matrix it was washed in with ten or more bed volumes of equilibrating buffer and then eluted with a linear gradient of NaCl in equilibrating buffer. Three to 7 ml eluate fractions were collected at a flow rate of 25 to 35 ml per hour. The absorbance (at 260 and 280 nanometers) of the eluate fractions was sampled with a Beckman Model DU spectrophotometer.

2.14 Enzyme assays

2.141 Determination of RNase activity

RNase activity was determined by measuring the increase in A260 of the acid-soluble ribonucleotides and oligoribonucleotides which are released as the degradation products of RNA. The standard reaction mixture contained final concentrations of .05 M buffer, .025% (w/v) (.25 mg/ml) Fleischmann's yeast s-RNA (in distilled H_2O), and various

amounts of tissue sample, in a total volume of 2.0 ml contained in heavy-walled pyrex Sorvall centrifuge tubes (catalog No. 119). Where necessary, the enzyme solution was diluted to ensure that the release of acid-soluble A260 was linear with incubation time. The reaction mixture was incubated for 30 or 60 minutes at 37° in a thermostatically controlled shaking water bath. The reaction was terminated by placing the [illegible] incubation tubes in a crushed ice bath and adding 2.0 ml ice-cold 1.4 N perchloric acid followed by thorough mixing. The assay tubes were allowed to stand 20 minutes in ice and then were centrifuged at 12,100 x g for 10 minutes at 0° to 4° in a type SS-34 rotar of a Sorvall RC2 centrifuge. The supernatant was carefully decanted and its absorbance at 260 nm measured immediately in silica cuvettes of 1 cm path length with a Beckman Model DU spectrophotometer. Appropriate controls without added enzyme and without added substrate were incubated and assayed concurrently in order to correct for any non-enzymatic depolymerization of RNA, the acid-soluble A260 material endogenous to the tissue sample, and the release of acid-soluble A260 from RNA endogenous to the tissue sample. Experimental assay samples were corrected by subtracting the total acid-soluble A260 released in controls, and the net RNase activity was expressed as the increase in A260 (ΔA260) per 30 minute incubation at 37°.

A unit of RNase activity was defined as the amount of enzyme which liberated acid-soluble material causing a net absorbancy increment of 1.0 O.D. 260 per ml per 30 minute incubation at 37°. Specific activity was expressed as units per mg protein.

2.142 Assay for deoxyribonuclease activity

The assay procedure for measuring DNase activity[291] was the same as that used for measuring RNase activity except highly polymerized double-stranded calf thymus DNA at a final concentration of .025% (w/v) was used as substrate. Incubation was for 60 minutes at 37°.

2.143 Assay for phosphodiesterase activity

Phosphodiesterase activity was measured by the liberation of p-nitrophenol from the synthetic substrate bis-(p-nitrophenyl) phosphate (Na).[292] The amount of p-nitrophenol released was detected by its absorbance at 400 nm.[293] The reaction mixture contained 33 mM buffer, 0.4 mM bis-(p-nitrophenyl) phosphate (Na), and enzyme sample in a total volume of 3.0 ml. Incubation was for 60 minutes at 37°. The reaction was terminated by transferring the incubation tubes to an ice bath and adding 3.0 ml of 0.04 N NaOH to each tube. The A400 was measured immediately.

2.144 Assay for pH 7.8 RNase inhibitor activity

RNase inhibitor activity was determined by measuring the reduction in the activity of a standard amount of bovine pancreatic RNase A in the presence of various amounts of inhibitor sample.[187] Crystalline bovine pancreatic RNase A (ca 50 Kunitz units per mg) was dissolved in 0.1% (w/v) purified gelatin solution to a concentration of 0.01 µg/ml. The standard RNase inhibitor assay contained .05 ml of the above stock solution (0.5 ng bovine pancreatic RNase A), 50 mM Tris-HCl pH 7.8, 1 mM EDTA diNa, various dilutions of tissue sample to give a level of inhibition between 30% and 70%, and .025% (w/v) sRNA in a total volume of 2.0 ml. The substrate was added last after mixing the previous components. Controls for the activity of 0.5 ng crystalline bovine pancreatic RNase A alone, and for the RNase activity of the tissue samples alone, as well as appropriate reagent blanks were incubated and assayed concurrently. Corrections for any RNase activity in the inhibitor samples were made in calculating the per cent inhibition of control pancreatic RNase A activity. Incubation was for 30 minutes at 37°. All other aspects of the assay procedure were the same as that previously described for the assay of RNase activity. The level of RNase inhibitor activity in the tissue samples was interpolated from experimentally constructed standard

curves relating per cent of control bovine pancreatic RNase A activity to protein concentration of the inhibitor sample. A unit of RNase inhibitor activity was defined as that amount of inhibitor which gave 50% inhibition of the control activity of 0.5 ng bovine pancreatic RNase A under these conditions and is hence numerically equivalent to the weight of bovine pancreatic RNase A inhibited.

2.15 Protein determination

Protein was measured according to the method of Lowry et al.[294] using bovine serum albumin as standard.

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III. EXPERIMENTAL RESULTS

3.0 Characteristics of the RNase Activities of Adult Rat Whole Brain Homogenates and Extracts

Initial experiments were aimed at detecting RNase activities in adult rat whole brain homogenates and extracts, and determining the effect on these activities of such variables as the medium used for homogenization of the tissue, and the pH, buffer, and ionic strength of the incubation medium. These experiments yielded information as to some general characteristics of the RNase enzyme system in brain and permitted selection of optimal conditions of assay for the RNase activities which were detected.

3.01 Effects of pH and buffer system on the RNase activities assayed in isotonic sucrose homogenates

Figure 1 shows three representative curves of the RNA-depolymerizing activity assayed at different pH values in freshly prepared isotonic sucrose homogenates of adult rat whole brain. RNase activity is detectable throughout the entire range of hydrogen ion concentrations tested with Tris-HCl buffer. Poorly defined activity maximae occur betwen pH 6 to 7 and between pH 8 to 9. Relative to the activity expressed in .05 M Tris-HCl, equimolar concentrations of imidazole-HCl buffer yield 65% higher activity

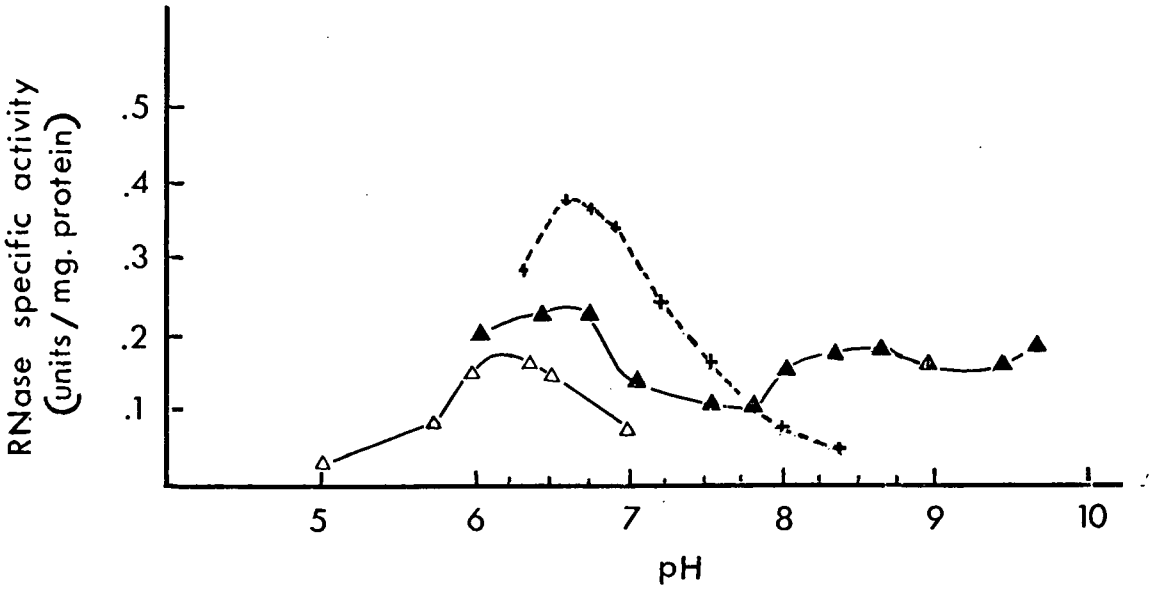

FIGURE 1. The effect of pH on the activity of RNase in isotonic sucrose homogenates of adult rat whole brain.

Aliquots of 0.1 ml were assayed from a homogenate containing 11.3 mg. protein/ml. Incubation was for 60 minutes. The buffer systems used were Tris-HCl —▲— , NH_4-acetate —△— , imidazole-HCl --+-- , and the final buffer concentration was 50 mM in all experiments unless stated otherwise.

at pH 6.6. Activity levels are about 30% lower with equimolar concentrations of NH_4-acetate buffer and the activity maxima is shifted to more acid pH values.

Since homogenization in isotonic sucrose is most likely to preserve the structural integrity of intracellular organelles it may be inferred that the level of RNase activity found under these conditions reflects the accessibility of the enzymes to the added substrate when the subcellular particles are still relatively intact. The amount of RNase activity expressed in isotonic sucrose homogenates is about 50, 76, and 68 per cent at pH 6.7, 7.8 and 9.5 respectively of the total extractable RNase activity assayed in 0.1% Triton X100 homogenates. Hence, it may be tentatively concluded that under conditions which most closely approximate the in situ state of these enzymes, more of the total detergent-extractable alkaline RNase activity (as compared to acid RNase) of brain is accessible to added substrate.

3.02 Solubilization of latent RNase activities by homogenization in 0.1% Triton X100

Homogenization of adult rat whole brain in 0.1% (v/v) of the non-ionic detergent Triton X100, which is known to disrupt and solubilize lipoprotein membranes, resulted in levels of RNase activity which were 50, 24, and 32 per cent higher at pH 6.7, 7.8, and 9.5 respectively than that expressed in isotonic sucrose homogenates. The 0.1% Triton

homogenate and the 0.1% Triton extract prepared from it according to the procedure described in section 2.11 of Methods exhibited nearly congruent pH versus RNase activity curves. Ninety-five per cent of the total RNase activity measured in Triton homogenates was consistently recovered in the extracts. This observation indicates that homogenization in 0.1% Triton X100 was effective in producing complete cell breakage and in releasing in soluble form any active RNase bound to or sequestered within sedimentable intracellular particles.

The alkaline to acid RNase activity ratio is considerably lower for the 0.1% Triton X100 extract (see Fig. 2 on page 72) compared to that for the isotonic sucrose homogenate, thus indicating that homogenization of the tissue in detergent as compared with isotonic sucrose resulted in the extraction of relatively more acid RNase activity than alkaline RNase activity. The large difference between the levels of measurable RNase activity under these two homogenization conditions suggests that a large fraction of the total RNA-degrading capacity of brain cells may be present <u>in situ</u> in an inactive or latent state adsorbed to or compartmentalized within intracellular organelles. However, these results do not permit any statement as to the relative contribution of these two possible <u>in situ</u>

enzyme states to the observed latency in enzyme activity. Also, no study was made of whether the ratio of RNase activity in sucrose homogenates varies with developmental age.

Triton most likely acts to release and activate those enzymes present in a latent, non-functional state by solubilizing intracellular lipoprotein membrane structures. The possibility that Triton-enhancement of expressed levels of RNase activity may be partially due to a more direct action on the enzyme molecules per se or to inactivation of inhibitors of RNase activities has not been excluded. A study of the effect of Triton X100 on the partially purified enzymes indicated 50% stimulation of acid RNase activity and no significant effect on alkaline RNases assayed at their respective pH optima (see Table VII, page 111. Since the effect of added Triton on the assay of RNase activity in more crude but soluble enzyme preparations (such as hypotonic buffer extracts prepared without detergent) was not determined, it must be considered that Triton may act at least in part by dissociating or preventing the formation of non-specific molecular aggregates involving RNase enzymes, and by minimizing the interaction of added substrate with non-specific proteins.

In summary, the preceding results clearly demonstrate the importance of ensuring complete disruption of intracellular membrane compartment if the full RNase capacity

of brain tissue is to be measured. Investigators of the components of the RNase enzyme system in rat liver have reported similar observations in that maximal RNase activity could only be detected upon disruption of intracellular organelle membranes by hypotonic shock; sonication,[296] repeated freeze-thaw cycles,[196,214] or non-ionic detergent treatment.[204,295] Rahman,[204] for instance, found that acid RNase in rat liver isotonic sucrose homogenates exhibited only 5 to 10% of the total acid RNase activity extractable with 0.1% Triton X100. He also reported that RNase activities assayed at both pH 7.8 and pH 9.5 were equally activated about 60% by 0.1% Triton X100.

3.03 Effects of pH, buffer system, ionic strength, and NaCl on RNase activities assayed in 0.1% Triton X100 extracts

Buffer-dependent differences in the activity and pH optimum of RNA-depolymerizing enzymes assayed in a freshly prepared 0.1% Triton X100 extract of adult rat whole brain are shown in Figure 2. The pH optimum of the acid RNase activity varies with the buffer system used. A distinct pyramidal activity maxima is observed at pH 6.7 with both imidazole-HCl and Tris-HCl buffers, and at pH 6.4 and 5.9 with NH_4-acetate and Na-phosphate buffers respectively.

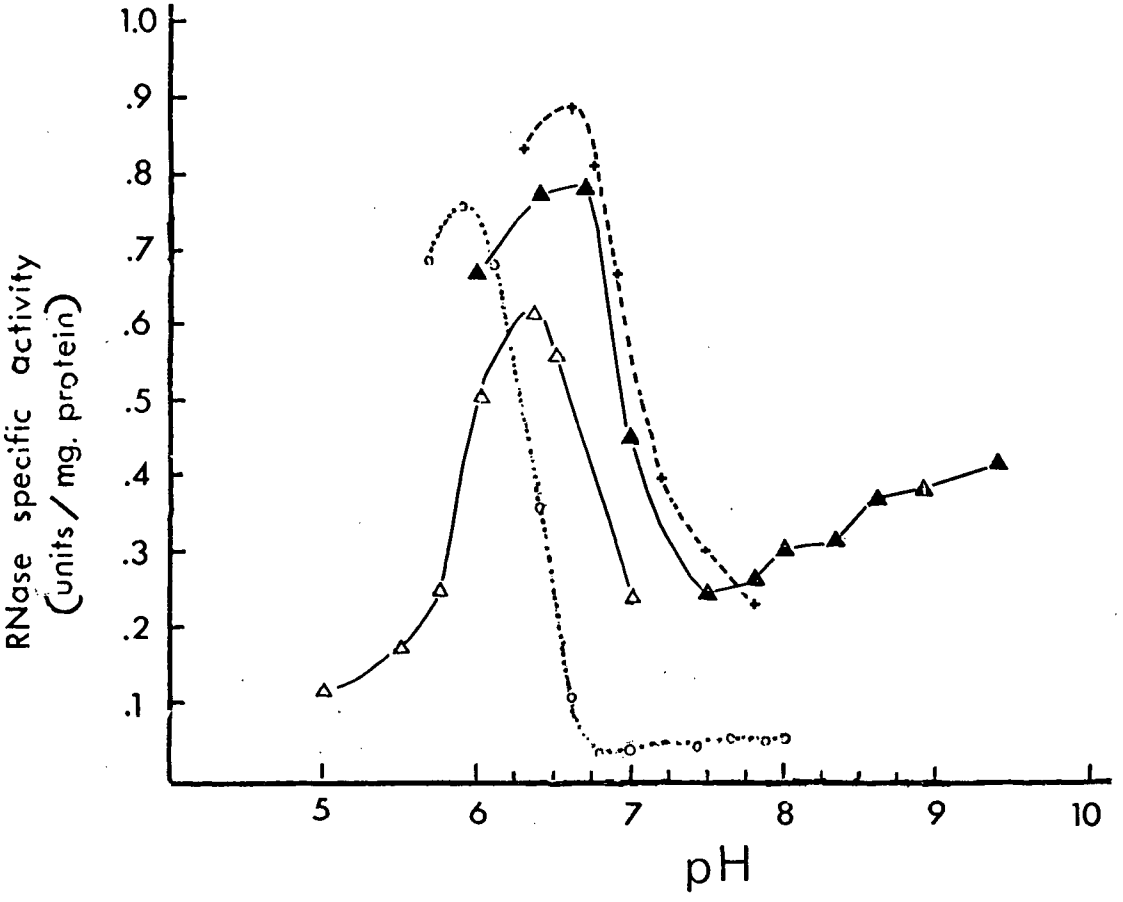

FIGURE 2. The effect of pH on the activity of RNase in 0.1% (w/v) Triton X100 extracts of adult rat whole brain.

Aliquots of 0.1 ml of a freshly prepared extract containing 5.2 mg. protein/ml were assayed in various buffer systems as a function of the hydrogen ion concentration of the incubation mixture. Incubation was for 60 minutes. The buffer systems used were Tris-HCl —▲— , NH_4-acetate —△— , imidazole-HCl --+-- , and Na-phosphate --o-- .

Of the various buffers tested, maximal acid RNase activity was obtained with imidazole-HCl. Tris-HCl and NH_4-acetate buffers yielded activities which were about 87% and 69% respectively of that obtained with imidazole-HCl. The level of activity at their respective pH optimae is nearly identical in equimolar concentrations of Tris-HCl and Na-phosphate buffers. However, in Na-phosphate buffer there occurs with decreasing hydrogen ion concentration a rapid fall to very low levels of activity by pH 6.7.

The concentration of the buffer was found to have a considerable effect on both acid RNase activity and alkaline RNase activity assayed at pH 7.9 (Table I). The activity of acid RNase declines and a shift to more acid pH optima occurs with increasing ionic strength (Fig. 3). The higher ionic strength of an equimolar concentration of Na-phosphate buffer, as compared with the monovalent buffer systems tested, may thus account for the more acid pH optimum obtained with this buffer.

In view of the dependence of acid RNase activity and pH optimum upon ionic strength, it seems likely that apparent discrepancies in the maximal activity and pH optimum reported by several workers for this enzyme in other mammalian tissues is due to differences in the ionic strength under which activity was assayed. Thus, for example, Rahman[204] reported a pH optimum of 5.5 for acid RNase from rat liver homogenate assayed in 200 mM acetate buffer.

TABLE I. EFFECT OF BUFFER CONCENTRATION ON RNASE ACTIVITY OF 0.1% TRITON X100 EXTRACTS OF ADULT RAT WHOLE BRAIN

pH	Buffer system	Final buffer concentration (mM)	Increase in A260 per 60 minute incubation
6.4	Na-phosphate	20	.549
6.4	Na-phosphate	50	.369
7.9	Na-phosphate	20	.167
7.9	Na-phosphate	50	.056
7.8	Tris-HCl	50	.266

A similar interdependence of ionic strength and pH optimum has been observed with bovine pancreatic RNase A[297] and bovine brain acid DNase.[298] Low ionic strength and low pH favor the denaturation of polynucleotide substrates. Increasing the salt concentration or ionic strength, on the other hand, by shielding the mutually repelled charged phosphate groups of the polynucleotide chain, stabilizes the hydrogen-bonded secondary structure and favors a more tightly coiled double-stranded configuration. Thus, the inhibitory effect on enzyme activity of increasing ionic strength may be partially, though not completely, counteracted by concurrently decreasing the pH, thereby maintaining a single-stranded form of the polynucleotide substrate. Assuming this to be the primary mechanism of action of salt, it can be inferred that acid RNase, like bovine pancreatic RNase A, preferentially attacks single-stranded sequences of RNA.

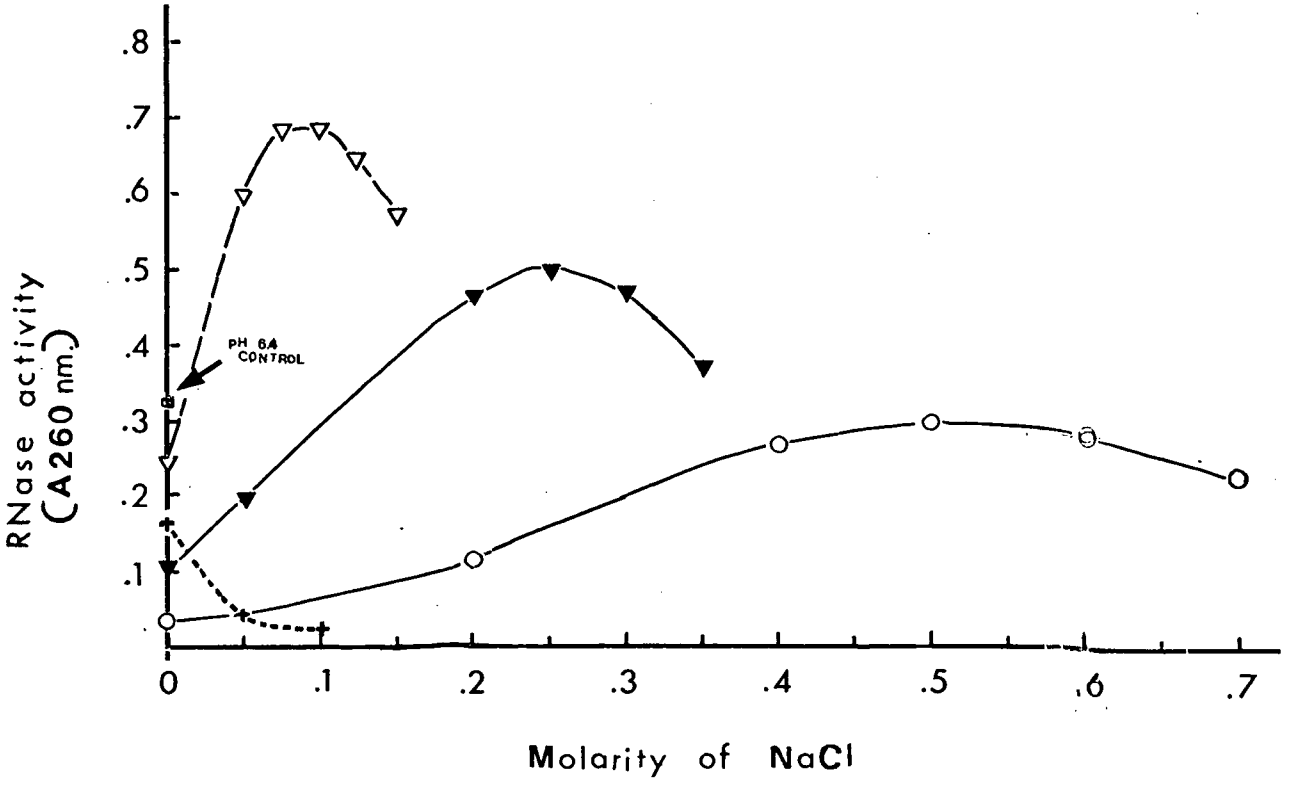

FIGURE 3. The effect of NaCl on acid RNase activity.

Aliquots of 0.1 ml of 0.1% Triton X100 extracts of adult rat whole brain were assayed (30 minute incubation) at the following pH: NH4-acetate, pH4 —o— ; NH4-acetate, pH5 —▼— ; NH4-acetate, pH6 —▽— ; Tris-HCl, pH7 --+-- . The activity assayed in Tris-HCl at pH 6.4 in the absence of added NaCl is also shown ▣ . RNase activity is expressed as the increase (corrected for substrate and enzyme blanks) in A260 per 30 minute incubation.

In a single experiment, it was indeed found that the activity of a partially purified acid RNase preparation was 30% higher with heat-denatured rat brain nuclear RNA than with native nuclear RNA. However, until this putative explanation is more conclusively tested, the direct effect of ionic strength on enzyme conformation, stability and solubility must also be taken into consideration in attempting to account for the observed ionic-strength-dependent changes in the activity and pH optimum of acid RNase.

In the alkaline region, the pH-RNase activity profile obtained with Tris-HCl buffer (Fig. 2) has a composite pattern with the appearance of several superimposed curves between pH 7.5 and 9.5. This profile is suggestive of the presence of at least two alkaline RNase activities with overlapping pH curves.

Phosphate and imidazole buffers yield very low activities in the alkaline pH range within which they retain effective buffering capacity. This is possibly due to the known[213] chelating activity of these buffers which may stabilize the pH 7.8 RNase-inhibitor complex or prevent the inactivation of RNase inhibitor by metal ions. Such an interpretation is suggested by the observations that (1) EDTA does not appreciably suppress RNase activity assayed at pH 7.8 in Na-phosphate buffer, and (2) the level of RNase activity assayed at pH 7.8 in the presence of 0.2 mM pCMB is the same in both Tris-HCl

and Na-phosphate buffers (Table II). Alternatively phosphate and imidazole ions may interact with the alkaline RNase, whose activity is normally expressed in this pH range when assayed with Tris-HCl buffer, to produce a less active conformation of the enzyme molecule. It may be worth noting for the purpose of comparison that bovine pancreatic RNase A, a basic protein with pI=7.8, is known[299] to have a strong affinity for multivalent anions such as orthophosphate.

TABLE II. EFFECT OF pCMB AND EDTA ON RNASE ACTIVITY ASSAYED IN 0.1% TRITON X100 EXTRACTS OF ADULT RAT WHOLE BRAIN

pH	Buffer system*	Reagent present at final concentration	Increase in A260 per 60 minute incubation
7.8	Tris-HCl		.266
7.9	Na-phosphate		.049
7.9	Na-phosphate	EDTA (1.0 mM)	.029
7.9	Na-phosphate	EDTA (2.0 mM)	.044
7.8	Tris-HCl	pCMB (0.2 mM)	.687
7.9	Na-phosphate	pCMB (0.2 mM)	.692

*Final buffer concentration in all cases was 50 mM.

Several other buffer systems (Tes-HCl, glycine-NaOH, and Na_2CO_3-$NaHCO_3$) with effective buffering capacities in the alkaline pH range were found to yield very low RNase activities compared with Tris-HCl.

3.04 Evidence indicating the presence of a protein inhibitor of pH 7.8 RNase activity in brain

3.041 Time-dependent activation of pH 7.8 RNase activity in stored enzyme preparations

A time-dependent activation of RNase activity assayed at pH 7.8 was observed upon storage of freshly prepared enzyme preparations at 0°. RNase activity assayed at pH 7.8 reached a maximum after about 6 days storage of 0.1% Triton X100 extracts at 0°, and subsequently remained relatively constant up to 14 days. Activity after 6 days storage at 0° was 86% greater than the activity assayed in freshly prepared extracts. Figure 14 on page 138 shows the pH versus RNase activity profiles of a cytosol fraction assayed immediately upon preparation and after two weeks storage at 0°. Maximal activation of RNase activity is observed at about pH 8.3 and the activity at this pH is 476% above that of freshly prepared cytosol. A 10% increase in RNase activity assayed at pH 6.7 also occurred in the 14 day old cytosol.

3.042 Inhibition of bovine pancreatic RNase A activity by brain extracts

Freshly prepared extracts inhibit bovine pancreatic RNase A activity and the degree of inhibition is proportional to the amount of extract added. Parachloromercuribenzoate prevented the inhibition of bovine pancreatic RNase A activity by freshly prepared extracts.

3.043 Activation by pCMB of RNase activity assayed at pH 7.8

The sulfhydryl blocking reagent, parachloromercuribenzoate (pCMB), activates RNase activity in freshly prepared extracts assayed at pH 7.8 and the degree of activation is positively correlated with the extract's capacity to inhibit bovine pancreatic RNase A activity. The time-dependent decline in the capacity of extracts to inhibit bovine pancreatic RNase A activity was accompanied by a concomitant decline in the capacity of pCMB to stimulate the endogenous pH 7.8 RNase activity.

The effects of pCMB are most likely explained by (1) its inactivation of a labile endogenous protein inhibitor which depends on the integrity of free sulfhydryl groups for its activity, and (2) the release of active pH 7.8 RNase from a RNase-inhibitor complex. The protein nature of the inhibitor is indicated by the facts that it is completely inactivated by heating at 100°, it does not pass through dialysis membranes, and it is inactivated by pCMB. The instability of the inhibitor is indicated by the time-dependent increase in pH 7.8 RNase activity observed upon storage of extracts and the concomitant decrease in pCMB stimulation of pH 7.8 RNase activity in stored extracts.

The time-dependent activation of pH 7.8 RNase activity during storage of extracts could be prevented by 1.0 mM EDTA

apparently due to the stabilization of RNase inhibitor by this reagent. The inhibitor activity could also be stabilized by β-mercaptoethanol or dithiothreitol. These sulfhydryl reducing agents were subsequently found to have no detectable effect on the partially purified pH 7.8 RNase activity (see Table VI, page 106).

3.044 Effect of EDTA on RNase activity assayed at pH 7.8

The effect of removing traces of heavy metal ions was examined by adding the chelating agent, EDTA, to incubation mixtures of freshly prepared extracts. EDTA was found to produce near total inhibition of RNase activity assayed at pH 7.8 in 50 mM Tris-HCl buffer. To determine whether this inhibition was due to augmentation of RNase inhibitor activity or to the binding of divalent cations more directly essential for RNase function, pH 7.8 RNase activity of extracts was measured in the presence of both 1.0 mM EDTA and 0.2 mM pCMB (Table III). The level of pH 7.8 RNase activity obtained under these conditions was slightly greater than that measured in the presence of 0.2 mM pCMB alone. The fact that pCMB can completely restore the pH 7.8 RNase activity inhibited by EDTA, combined with the observation (Table VI) that neither pCMB nor EDTA altered the activity of partially purified pH 7.8 RNase preparations indicates that the effect of these reagents is primarily upon the activity of RNase inhibitor. EDTA thus appears to act by liberating RNase

inhibitor from inactive complexes with traces of metal ions, the free inhibitor thereby becoming available to combine with free pH 7.8 RNase. A corollary of this explanation of the mechanism of EDTA action is that inhibition of bovine pancreatic RNase A by the inhibitor endogenous to extracts is greater in the presence of EDTA than in its absence (Table IV).

TABLE III. ACTIVATION OF EDTA-INHIBITED DEAE-CELLULOSE ELUATE pH 7.8 RNase ACTIVITY BY pCMB

Enzyme preparation* added (mls)	Reagent present at final concentration	Increase in A260 per 30 minute incubation
.05		.066
.05	EDTA (0.5 mM)	.010
.05	EDTA (5.0 mM)	.035
.05	pCMB (0.2 mM)	.928
.05	EDTA (0.5 mM)+ pCMB (0.2 mM)	.974
.05	EDTA (5.0 mM)+ pCMB (0.2 mM)	1.110

*The pooled DEAE-cellulose eluate pH 7.8 RNase activity (fractions 47-65 in Fig. 8) was dialyzed against 20 mM Tris-HCl, pH 7.4 buffer and aliquots of the dialysate were assayed in 50 mM Tris-HCl, pH 7.8 buffer.

TABLE IV. ENHANCEMENT OF RNase INHIBITOR ACTIVITY BY EDTA

Addition to assay			Increase in A260 per 30 minute incubation
0.5 ng	pancreatic RNase A		.703
0.5 ng	pancreatic RNase A	EDTA (1.0 mM)	.703
	cytosol*		.043
	cytosol*	EDTA (1.0 mM)	.009
0.5 ng	pancreatic RNase A	cytosol*	.493
0.5 ng	pancreatic RNase A	cytosol* EDTA (1.0 mM)	.358

*Aliquots (0.15 ml) of a 1/10 dilution (in ice-cold .02 M Tris-HCl, pH 7.8 buffer) of a cytosol fraction freshly prepared from adult rat whole brain were assayed. Assay conditions were as described in section 2.144 of Methods.

3.045 Comparison of the effects of pCMB on RNase activities in liver and brain

A comparison of the level of inhibitor-bound RNase in the brain and liver of adult rats was made by measuring the capacity of pCMB to stimulate the RNase activity from each organ. This date is shown in Figures 4 and 5. A plot of the RNase activity as a function of pH showed that in the absence of pCMB the specific activity of acid RNase and alkaline RNase of liver was 3-fold and 5-fold greater respectively than that of brain. When total pH 7.8 RNase

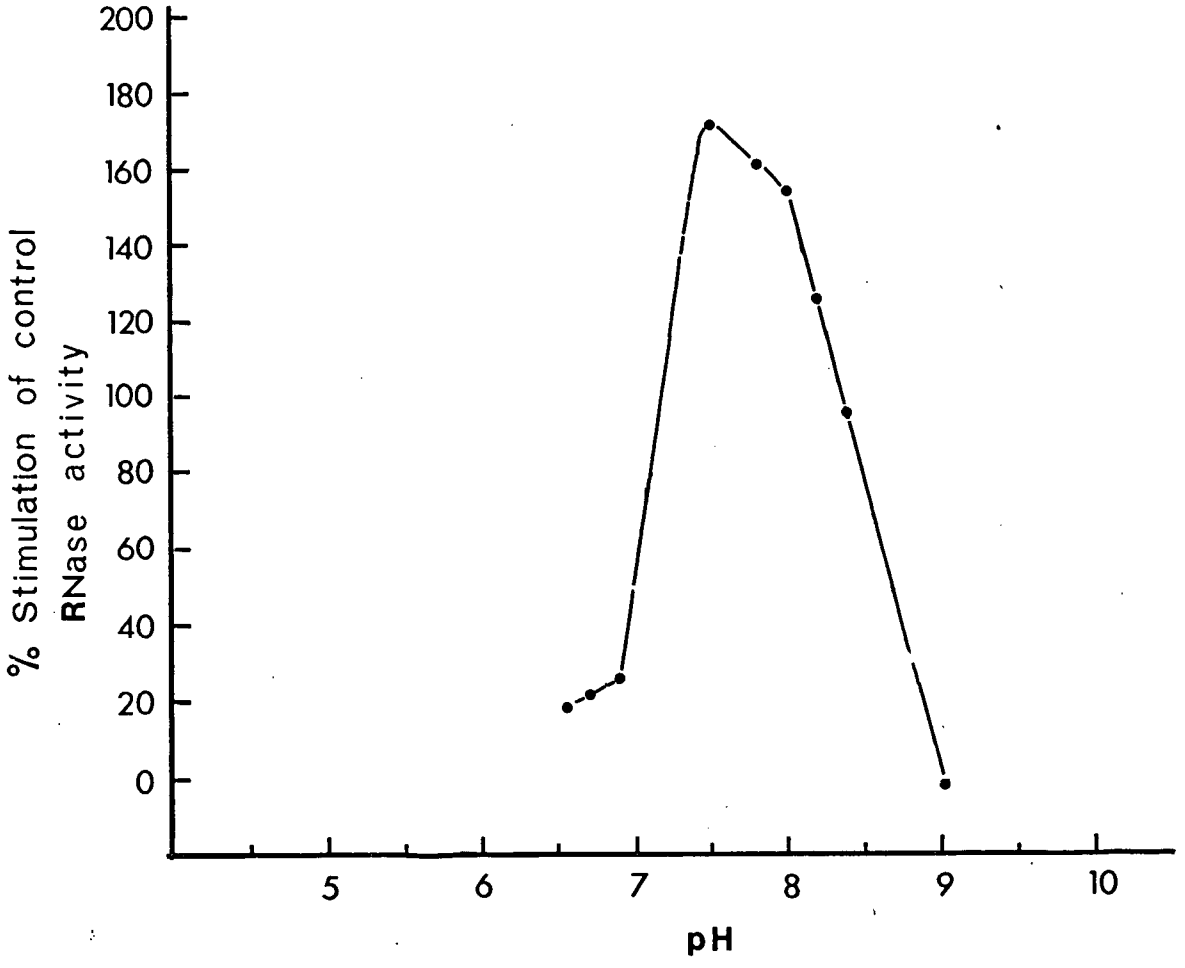

FIGURE 4. The stimulation by pCMB of RNase activity in 0.1% Triton X100 extracts of adult rat whole brain.

Aliquots of 0.5 ml of extract were incubated for 60 minutes in 50 mM Tris-HCl buffer. Per cent stimulation was calculated as the difference in A260 between samples assayed at a given pH with and without 0.1 mM pCMB divided by the control activity in the absence of pCMB.

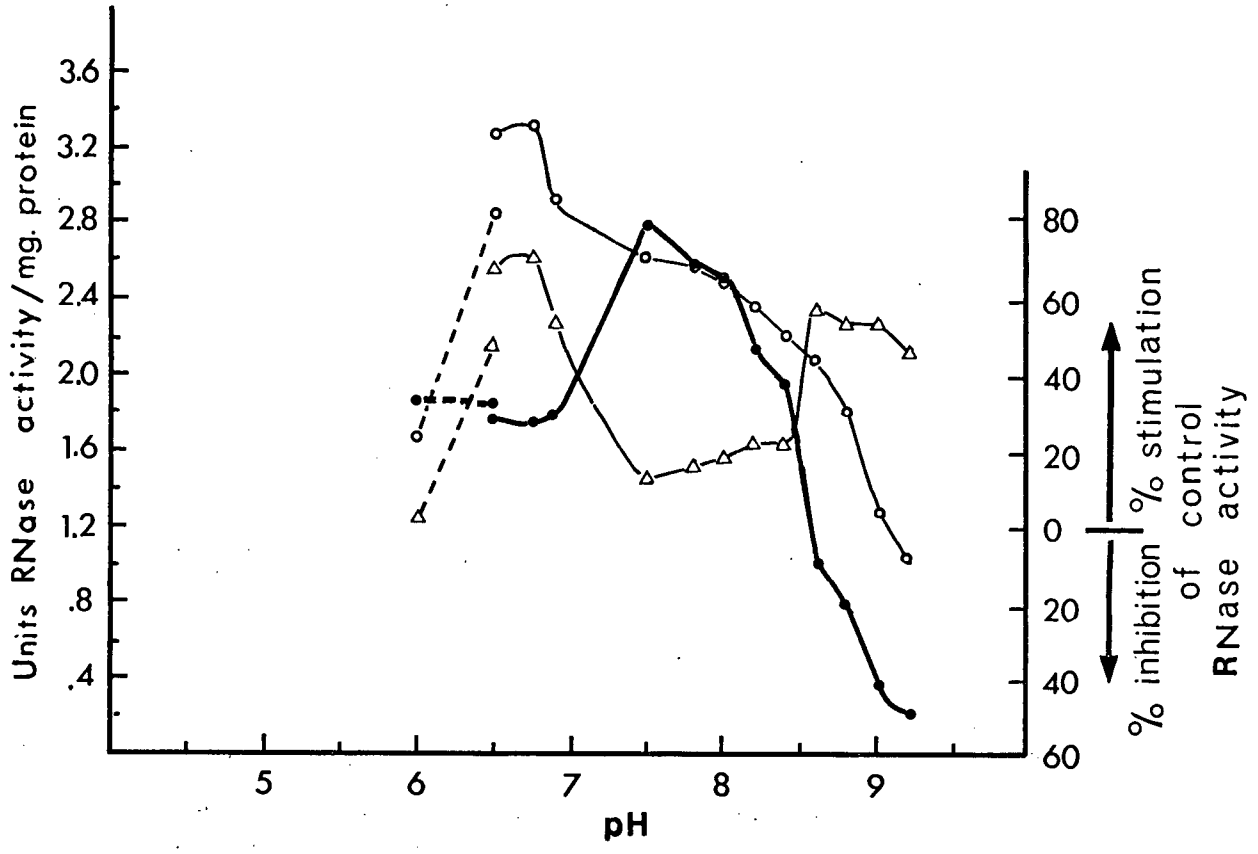

FIGURE 5. The effect of pCMB on RNase activity in 0.1% Triton X100 homogenate of adult rat liver.

Aliquots of 0.05 ml of a 5% (wet weight/liver/final volume) homogenate were incubated for 30 minutes. The RNase activity, expressed in units/mg protein, was assayed at each pH without pCMB (NH_4-acetate buffer --△--, Tris-HCl buffer —△—) and in the presence of 0.1 mM pCMB (NH_4-acetate buffer --○--, Tris-HCl buffer —○—). The difference curve, —●—, is expressed as per cent stimulation and per cent inhibition by pCMB of control RNase activity.

activity was assayed in the presence of 0.1 mM pCMB, the specific activity was about 3-fold greater for liver than brain. The activity released by pCMB was, however, 95% greater in brain than liver. In both organs, pCMB activation was greatest around pH 7.5. The pH-dependent effect of pCMB may be due to changes in the reactivity and/or accessibility to pCMB of the critical sulfhydryl groups at different pH.

Thus, in addition to stimulating enzyme activity, pCMB produces a change in the shape of the pH-RNase activity curve. This may be related to the observation of Colter et al.[216] who mixed various dilutions of RNase inhibitor with a standard amount of bovine pancreatic RNase A. The pH-activity curve of the uninhibited enzyme had a sharp optimum at pH 7.8. As the ratio of RNase inhibitor to pancreatic RNase A increased, the position of the optimum shifted to more alkaline pH values. Hence, the apparent activity optimum at about pH 8.5 observed in isotonic sucrose homogenates (Fig. 1) and 0.1% Triton X100 extracts (Fig. 2) of brain assayed without pCMB may be due to the pH-dependence of RNase-inhibitor complex formation—more uninhibited RNase activity being expressed at more alkaline pH due to dissociation of inhibitor-bound pH 7.8 RNase.

Between pH 6 and pH 7, pCMB consistently stimulates RNase activity in extracts by 20 to 30%. This stimulation

is not due to a direct effect on acid RNase, nor is it due to release of latent acid RNase from an inhibitor-bound form, since acid RNase activity assayed at all stages of purification subsequent to its separation from alkaline RNase activity was inhibited 30% by the same concentrations of pCMB (see Figure 6a, page 92). Rather, this activation most likely represents the net outcome of direct inhibition of acid RNase activity by pCMB and the release from inhibition of pH 7.8 RNase activity which retains 68% of its activity when assayed at pH 6.7 (see Fig. 9c, page 104). A related observation has been reported by Rahman[204] who observed a 15 to 20% inhibition of RNase activity in the acid pH range upon reconstituting the supernatant and sedimentable particulate fractions of rat liver isotonic sucrose homogenates.

The inhibitory effect of pCMB above pH 8.7 is also likely to be a composite result. The expected increase in residual pH 7.8 RNase activity normally expressed at this pH upon release from inhibitor is masked by the strong direct inhibitory effect of the sulfhydryl blocking reagent upon some other alkaline RNase activity in this pH range. This inhibition is significantly greater for liver than for brain.

Maximal activation of RNase activity in brain extracts assayed in the pH range between 7.0 and 8.5 was obtained at pCMB concentrations of 0.2 mM. Higher concentrations of

pCMB had no further effect, suggesting that pCMB has no direct stimulatory or inhibitory effect on the pH 7.8 RNase activity per se. This was subsequently confirmed by measuring the effect of pCMB on partially purified pH 7.8 RNase activity (see Table VI, page 106).

3.05 Summary comment on the variables influencing the determination of RNase activities in crude extracts

The literature shows a marked lack of agreement between workers [193,202,214,300] who estimated the levels of acid RNase and pH 7.8 RNase activities in rat liver. In this organ, the pH 7.8 RNase to acid RNase activity ratio obtained by Roth[300] was 0.2; Shortman[214] obtained a ratio for frozen-thawed homogenates of 0.05, and De Lamirande and Allard[202] obtained a ratio of 2.3. In the present study a pH 7.8 RNase to acid RNase activity ratio of 0.3 was obtained for brain extracts, as compared to 0.7 when 0.1 mM pCMB was present in the assay. It is apparent from the present study that a wide spectrum of results could be obtained ranging from no RNase inhibitor and high pH 7.8 RNase activities to high inhibitor and no pH 7.8 RNase activity depending on the care taken to avoid inhibitor inactivation in the course of homogenate preparation and assay. Also, as previously mentioned, differences in such parameters as buffer system, ionic strength, and the extent of rupture of subcellular organelles do affect the

apparent level of RNase activities measured *in vitro*. Yet another reason for the marked discrepancies in the absolute values and activity ratios obtained by different investigators is the lack of standardization of the RNA-precipitating agent used to stop the reaction.[202] Thus, it is often difficult to compare the results reported by different investigators since in the absence of common bases of reference no reliable basis for comparison exists.

3.1 Separation and Partial Purification of the Components of the Multi-enzyme-Inhibitor System of Adult Rat Whole Brain 0.1% Triton X100 Extracts

The preceding experiments revealed the presence in rat whole brain homogenates and extracts of at least two ribonucleases distinguishable by the pH at which they exhibit optimal activity. The alkaline RNase activity referred to as pH 7.8 RNase appears to be present largely in a latent form bound to a protein inhibitor.

Although it is important to characterize enzyme activities under conditions which most closely approximate their *in situ* state in order to permit extrapolation of *in vitro* experimental results to the *in vivo* condition, the heterogeneity of RNase activities present in crude extracts of rat brain complicates the interpretation of results obtained using such a complex mixture of molecular components. An attempt

was, therefore, made to separate the components of the RNase enzyme system from each other and from other proteins by differential precipitation with ammonium sulphate followed by anion exchange column chromatography of the ammonium sulphate precipitable fractions.

3.11 Ammonium sulphate fractionation of 0.1% Triton X100 extracts of adult rat whole brain

Triton X100 (0.1%) extracts of adult rat whole brain were fractionated with powdered ammonium sulphate according to the procedure described in section 2.131 of Methods. The ammonium sulphate fractionation data recorded in Table V shows that this procedure effected a clear separation of acid and alkaline RNase activities.

A considerable loss of pH 6.7 RNase activity occurs during the ammonium sulphate fractionation and subsequent removal of the salt by dialysis. Total per cent recovery of pH 6.7 RNase activity in all the dialyzed ammonium fractions was consistently about 60%. The protein fraction precipitating between 75 and 100% ammonium sulphate saturation contained the highest specific activity and accounted for nearly 70% of the total recovered pH 6.7 RNase activity.

TABLE V. RECOVERY OF RNase ACTIVITIES IN AMMONIUM SULPHATE PRECIPITABLE FRACTIONS*

Enzyme preparation	RNase activity assayed at pH 6.7		RNase activity assayed at pH 9.5		RNase activity assayed at pH 7.8		RNase activity assayed at pH 7.8 in the presence of 0.2 mM pCMB		Calculated latent RNase activity at pH 7.8	
	Total activity	% Recovery	Total activity	% Recovery	Total activity	% Recovery	Total activity	% Recovery	Total activity	% Recovery
0.1% Triton X100 extract	1,104.0	100	591.1	100	414.0	100	920.0	100	506.0	
0-25% saturated $(NH_4)_2SO_4$ fraction	13.2	1.2	15.4	2.6	11.7	2.8	10.4	1.1	0.0	0.0
25-55% saturated $(NH_4)_2SO_4$ fraction	137.5	12.5	687.8	116.4	389.6	94.1	948.6	103.1	559.0	110.5
55-75% saturated $(NH_4)_2SO_4$ fraction	65.4	5.9	28.9	4.9	2.7	0.6	42.4	4.6	39.7	7.8
75-100% saturated $(NH_4)_2SO_4$ fraction	424.2	38.4	69.8	11.8	2.0	0.4	51.5	5.5	49.5	9.8
% of initial activity recovered in all $(NH_4)_2SO_4$ fractions		58.0		135.7		97.9		114.3		128.1

*An initial volume of 230 ml 0.1% Triton X100 extract of adult rat whole brain was fractionated with $(NH_4)_2SO_4$ according to the procedure described in section 2.131 of Methods. After removal of the salt by dialysis against .02 M Tris-HCl, pH 7.4 buffer the fractions obtained were assayed in .05 M Tris-HCl buffer for RNase activity at pH 7.8 with and without 0.2 mM pCMB, and at pH 6.7 and pH 9.5. Latent pH 7.8 RNase activity is the difference in RNase activity at pH 7.8 assayed with and without pCMB.

RNase activity is expressed in units as defined in section 2.141 of Methods

Total recovery of pH 9.5 RNase activity was generally higher but somewhat more variable, ranging from 75 to 135%. About 85% of the total recovered pH 9.5 RNase activity as well as the highest specific activity was found in the ammonium sulphate fraction precipitating between 25 and 35% saturation.

Most of the free pH 7.8 RNase activity (assayed without pCMB) as well as the latent, inhibitor-bound pH 7.8 RNase activity (the difference between the total pH 7.8 RNase activity assayed in the presence of 0.2 mM pCMB and the free pH 7.8 RNase activity assayed without pCMB) was also recovered in the 25 to 55% saturated ammonium sulphate fraction. Total recoveries of both free and total pH 7.8 RNase activity ranged between 70 and 100%.

Because the capacity of different amounts of each ammonium sulphate fraction to inhibit the activity of a standard amount of bovine pancreatic RNase A was not measured, no quantitative statement can be made as to the amount of free RNase inhibitor activity recovered in each ammonium sulphate fraction. However, free RNase inhibitor activity appears to be diffusely distributed throughout fractions 25-55, 55-75, and 75-100% saturation since each of these was capable of inhibiting bovine pancreatic RNase A activity and this inhibition could be prevented by 0.2 mM

pCMB. This observation is consistent with Roth's report[211] that free RNase inhibitor of rat liver is precipitated mostly between 35-55% ammonium sulphate saturation with some inhibitor activity precipitating at greater than 60% saturation.

The fact that the total recovery of free pH 7.8 RNase activity was never greater than 100% suggests that the endogenous pH 7.8 RNase-inhibitor complex was not dissociated during ammonium sulphate fractionation. This indicates the firmness of the binding between inhibitor and RNase. Shortman has reported[213] that salt concentrations up to 0.3 M did not significantly weaken the interaction between rat liver RNase inhibitor and bovine pancreatic RNase A.

In summary, ammonium sulphate fractionation does not effect any resolution of the following molecular species: free pH 7.8 RNase, free RNase inhibitor, pH 7.8 RNase-inhibitor complex, and pH 9.5 RNase. However, it succeeds in removing considerable amounts of inactive protein and in separating the above components from the acid RNase activity (see Figs. 6a and 6b).

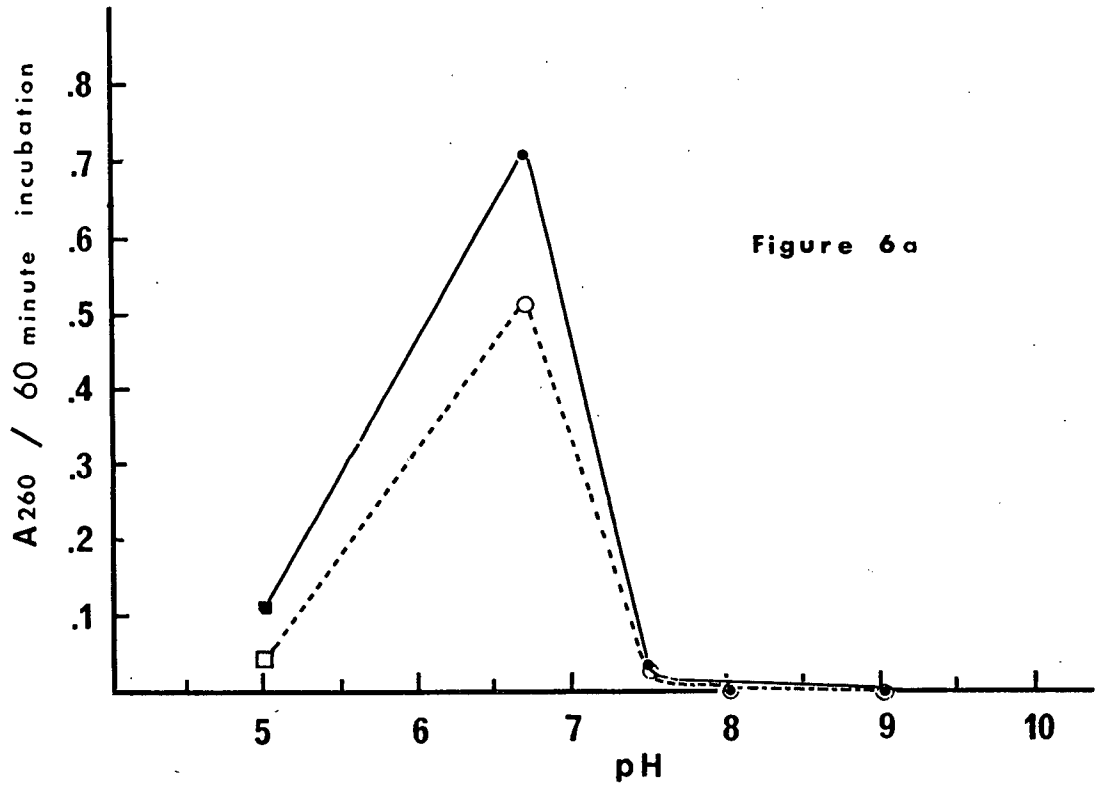

Figure 6a

FIGURE 6a. The effect of pH on the RNase activity precipitable between 75 and 100 per cent saturation with ammonium sulphate.

An initial volume of 160 ml of a 0.1% Triton X100 extract of adult rat whole brain was fractionated according to the procedure described in Materials and Methods. The protein fraction precipitating between 75-100% saturation of ammonium sulphate was dissolved in and dialyzed against 20mM Tris-HCl, pH 7.4 buffer and 20 µl aliquots of the dialysate were assayed in 50 mM buffer in the absence (NH4-acetate ■ ; Tris-HCl —•—) and in the presence (NH4-acetate □ ; Tris-HCl --○--) of 0.1 mM pCMB.

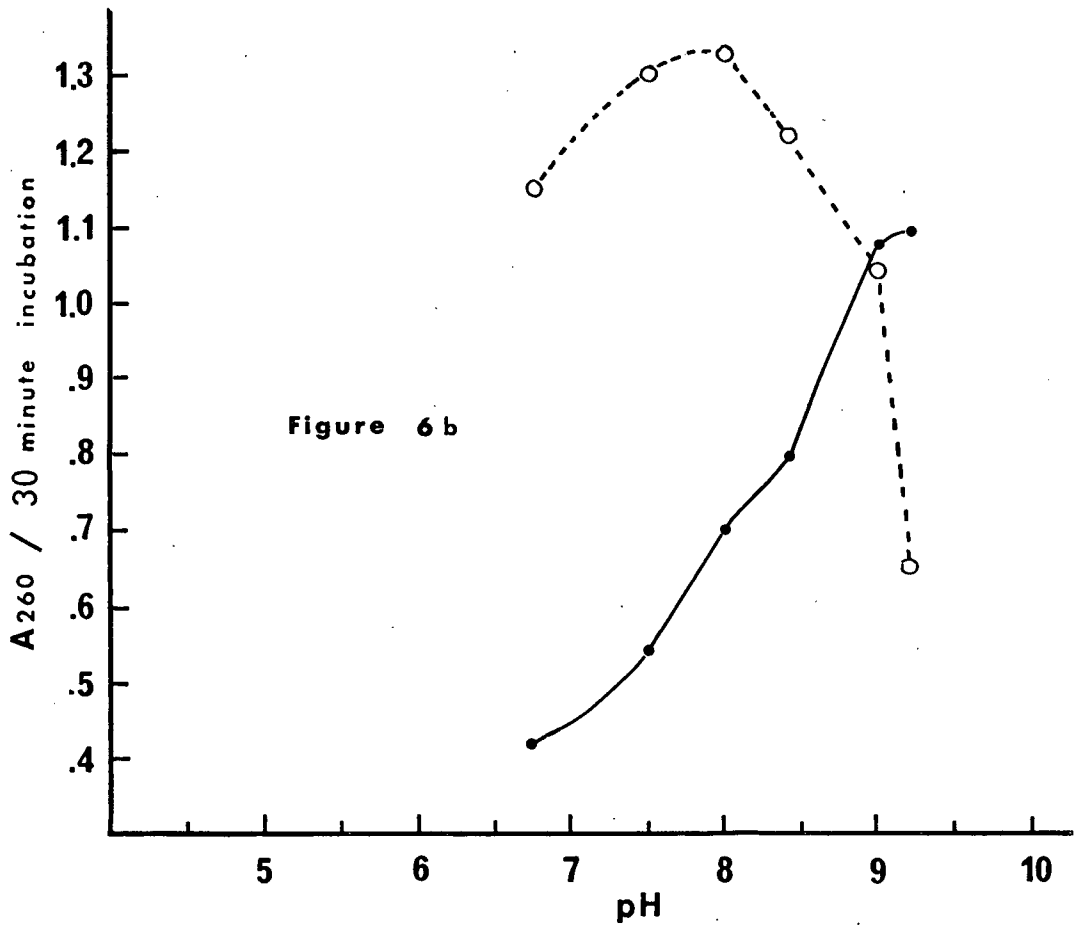

FIGURE 6b.The effect of pH on the RNase activity precipitable between 25 and 55 per cent saturation with ammonium sulphate.

The procedure for the preparation and assay of this fraction is the same as that described in the legend to Figure 6a, with the exception that 50 µls of the dialysate was used for assay and incubation was for 30 minutes. RNase activity was assayed in 50 mM Tris-HCl buffer in the absence —o— , and in the presence --o--- of 0.1 mM pCMB.

3.12 DEAE-cellulose column chromatography of the 25-55% and the 75-100% saturated ammonium sulphate fractions

An attempt was made to achieve further separation and purification of the constituent molecular species of the 75 to 100% and the 25 to 55% ammonium sulphate fractions by chromatography of each of these fractions on columns of DEAE-cellulose according to the procedure described in section 2.132 of Methods.

Figure 7 shows a typical elution profile of protein and pH 6.7 RNase activity upon chromatography on DEAE-cellulose of a 75-100% saturated ammonium sulphate fraction. Only one RNase activity peak, eluting between 0.1 and 0.2 M NaCl, was detected in the eluate fractions.

When the 25 to 55% saturated ammonium sulphate fraction was chromatographed on a column of DEAE-cellulose two peaks of RNase activity were detected in the eluate fractions (Fig. 8). The first enzyme peak coincides with the unadsorbed protein washed through the column with equilibrating buffer. The second enzyme peak was eluted at a NaCl concentration between 0.15 and 0.25 M. When the eluate fractions were assayed for their capacity to inhibit bovine pancreatic RNase A activity, a broad peak of inhibitor activity was detected eluting between 0.15 and 0.5 M NaCl and overlapping the free pH 7.8 RNase activity

Figure 7

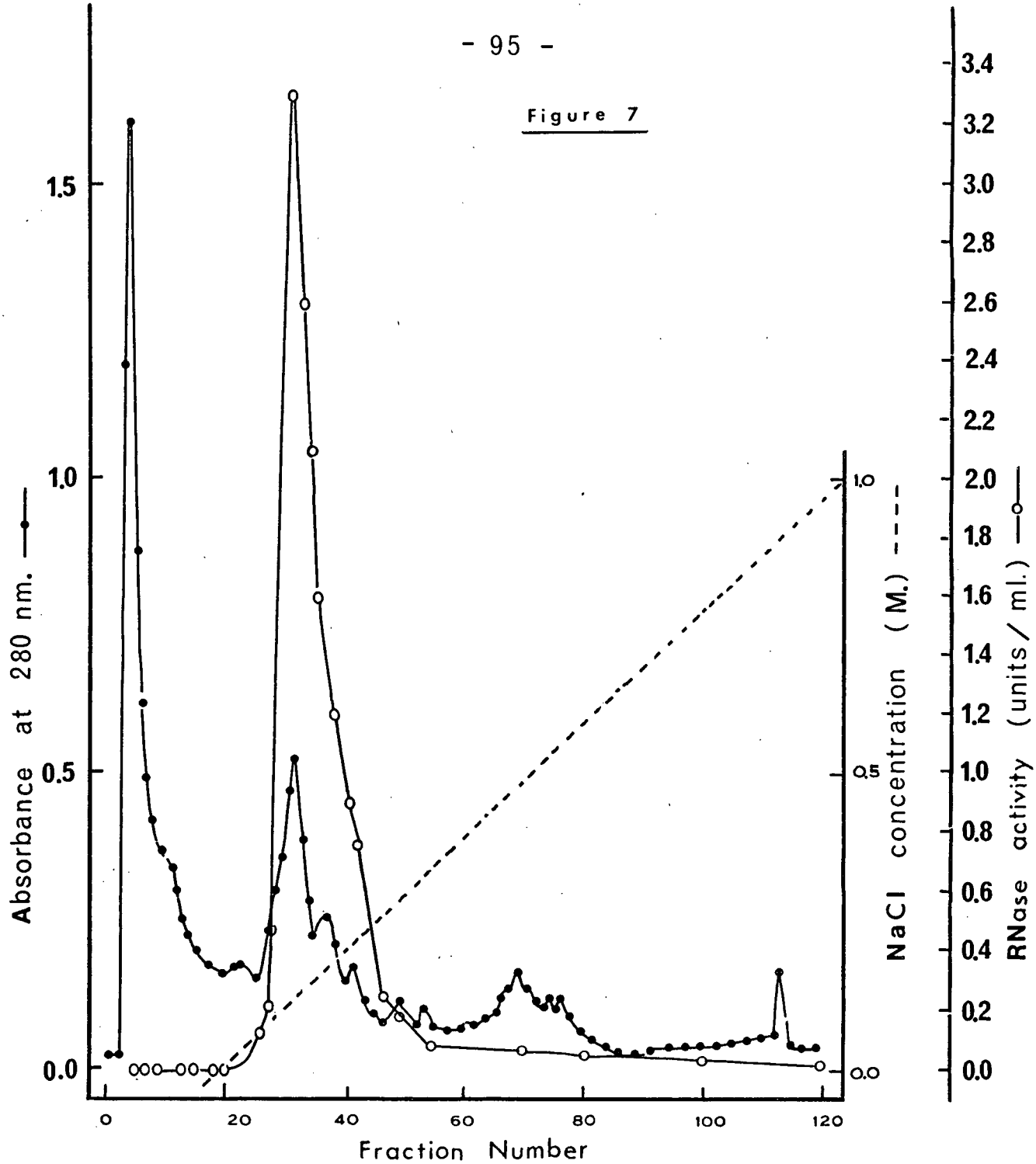

FIGURE 7. Elution profile of 75-100% saturated ammonium sulphate precipitable fraction chromatographed on DEAE-cellulose.

FIGURE 7. Elution profile of 75-100% saturated ammonium sulphate precipitable fraction chromatographed on DEAE-cellulose.

The protein fraction precipitating between 75 and 100 per cent saturation with ammonium sulphate was dialyzed against .02 M Tris-HCl, pH 6.9 buffer, and 4.0 mls of the dialysate containing 56.8 mg protein and 152 units of RNase activity assayed at pH 6.7 was loaded on a 1 cm by 15 cm column of DEAE-cellulose pre-equilibrated with 500 ml of .02 M Tris-HCl, pH 6.9 buffer. The sample was eluted with a linearly increasing salt gradient (250 ml Tris-HCl, pH 6.9 to 250 ml 1.0 M NaCl in .02 M Tris-HCl, pH 6.9). Fractions of 4.4 ml were collected. Aliquots (0.2 ml) of the undialyzed eluate fractions were assayed for RNase activity at pH 6.7 in 50 mM Tris-HCl buffer. Incubation was for 60 minutes.

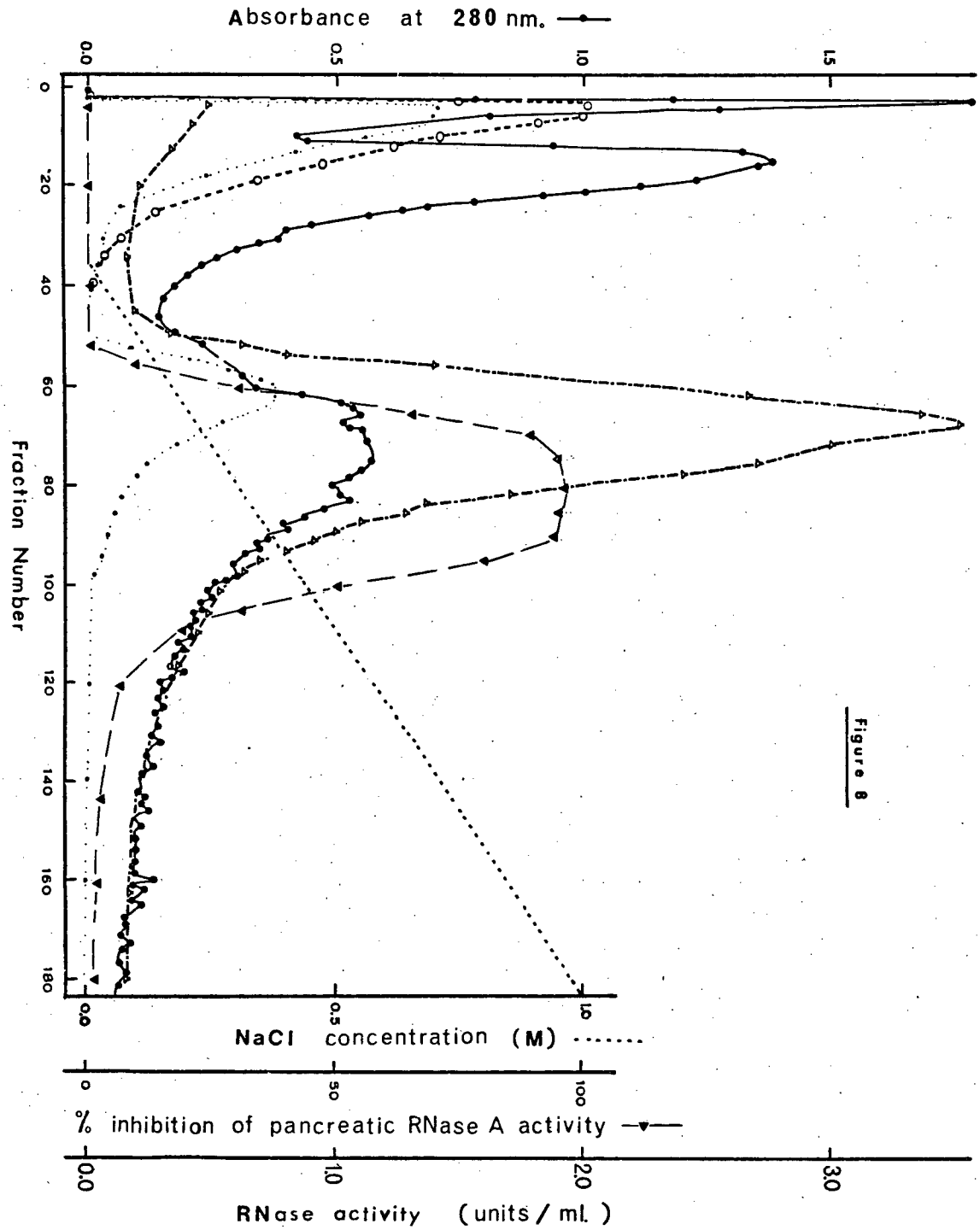

FIGURE 8. Elution profile of the 25-55% saturated ammonium sulphate precipitable fraction chromatographed on DEAE-cellulose.

FIGURE 8. Elution profile of the 25-55% saturated ammonium sulphate precipitable fraction chromatographed on DEAE-cellulose.

The protein fraction precipitating between 25 and 55 per cent saturation with ammonium sulphate was dialyzed against .02 M Tris-HCl, pH 6.9 buffer and 10 ml of the dialysate containing 393 mg of protein was loaded on a 1 cm by 19.5 cm column of DEAE-cellulose pre-equilibrated with .02 M Tris-HCl, pH 6.9 buffer. The sample was eluted with a linearly increasing salt gradient (400 ml .02 M Tris-HCl, pH 6.9 to 400 ml 1.0 M NaCl in .02 M Tris-HCl, pH 6.9). Fractions of 4.8 ml were collected. Aliquots (0.2 ml) of the undialyzed eluate fractions were assayed immediately for RNase activity at pH 9.5 --o-- and for free RNase inhibitor activity —▼—, and on the following day for free ····· and total (with 0.2 mM pCMB)--▲-- RNase activity at pH 7.8. Incubations in all cases were for 60 minutes. Other details of the assay procedure were as described for the standard assay condition in Materials and Methods.

assayed in the absence of pCMB as well as the total pH 7.8 RNase activity assayed in the presence of 0.2 mM pCMB.

The wash-through RNase activity was found to be very unstable and rapidly lost activity upon storage in the cold room (4°). This activity was inhibited by pCMB when assayed at pH 7.8 or pH 9.5. At a final conentration of 0.2 mM, pCMB produced 40% and 70% inhibition at pH 7.8 and pH 9.5 respectively. The fact that this RNase activity is not adsorbed on DEAE-cellulose columns equilibrated at pH 7.4 indicates that it has no net negative charge at this pH and is hence a basic protein.

The free pH 7.8 RNase activity recovered in the eluate fractions was up to 300% greater than the amount loaded on the column. This is inferred to be due to a preferential inactivation of the inhibitor component of pH 7.8 RNase-inhibitor complexes, as well as to the dissociation of such complexes and the partial separation of free pH 7.8 RNase activity from free inhibitor activity during the chromatography.

The recovery of total pH 7.8 RNase activity (assayed in the presence of 0.2 mM pCMB) in the eluate fractions ranged between 75 and 100% of the amount loaded.

The free RNase inhibitor activity in the eluate fractions was very unstable. Upon storage in the cold room,

it exhibited a rapid loss in its capacity to inhibit pancreatic RNase A activity. The per cent inhibition of bovine pancreatic RNase A activity was highest at pH 7.5, thus indicating that the free RNase inhibitor activity has a pH optimum of 7.5.

3.2 Properties of the Three Separated RNase Activities

Three apparently distinguishable RNase activities were thus separated from 0.1% Triton X100 extracts by means of ammonium sulphate fractionation followed by DEAE-cellulose column chromatography. In order to conclusively determine whether these three enzyme fractions were distinct enzymes and to further characterize their properties, the effect of various reagents on the activity of the DEAE-cellulose eluate enzyme fractions was studied. The characteristics of the pH 7.8 RNase reported in the following sections (Fig. 9c, Tables VI and VII) were determined using DEAE-cellulose eluate fractions which had been stored for some time and hence exhibited no RNase inhibitor activity. The absence of RNase inhibitor activity was indicated by the failure of pCMB to stimulate the RNase activity in these fractions and the inability of these enzyme fractions to inhibit bovine pancreatic RNase A activity.

3.21 Effect of pH

DEAE-cellulose eluate fractions with peak RNase activities were assayed at various hydrogen ion concentrations in 0.05 M buffer.

The pH optimum of the DEAE-cellulose eluate acid RNase activity was found to have shifted to a more acid pH, with maximal activity now occurring at pH 6.4 with Tris-HCl and at pH 6.0 with NH_4 acetate buffers (Fig. 9a). This activity was free of contamination by pH 7.8 RNase as indicated by the fact that this enzyme fraction exhibits no activity when assayed at pH 7.8.

The DEAE-cellulose wash-through RNase activity exhibits maximal activity above pH 9 and retains 56% of activity when assayed at pH 7.8. The shoulder in the pH curve between pH 8.0 and 8.5 suggests that this enzyme fraction may consist of a mixture of more than one distinct enzyme species. Further purification is required to test this possibility.

The pH curve of the DEAE-cellulose eluate pH 7.8 RNase activity is shown in Figure 9c. This enzyme exhibits a broad but symmetrical pH profile with a pH optimum of about 7.7. It retains 68% of its activity when assayed at pH 6.7.

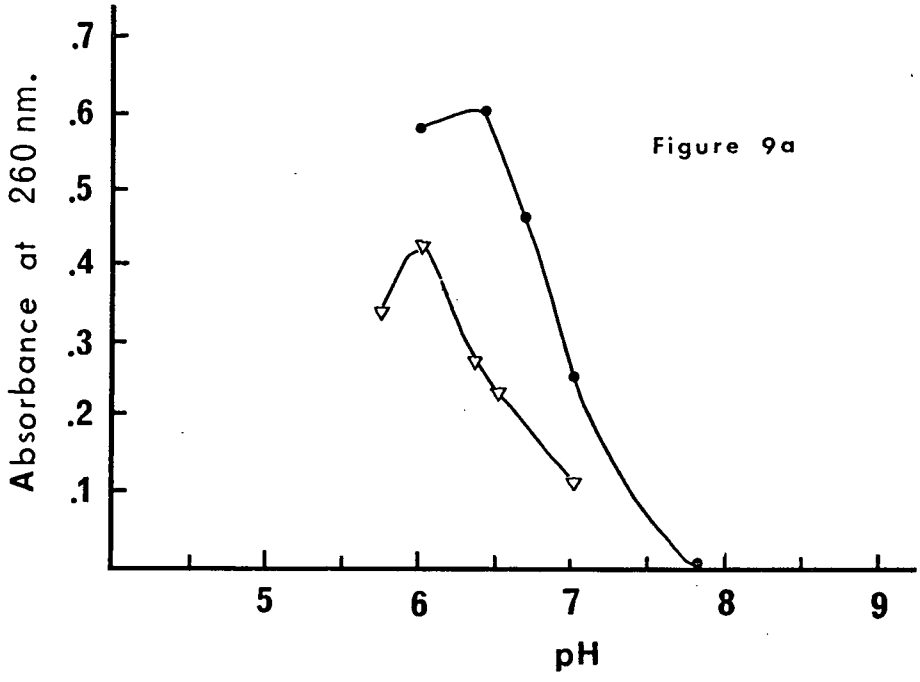

FIGURE 9a. The effect of pH on DEAE-cellulose eluate acid RNase activity.

Peak activity fractions eluting between 0.1 and 0.25 M NaCl in Figure 7 were pooled, dialyzed against .02 M Tris-HCl, pH 7.4 buffer, and the dialysate was brought to 30% (v/v) glycerol. Aliquots (0.2 ml) of this final preparation were assayed at various pH in .05 M NH4-acetate —▽— , or in .05 M Tris-HCl —•— buffers. Incubation was for 60 minutes.

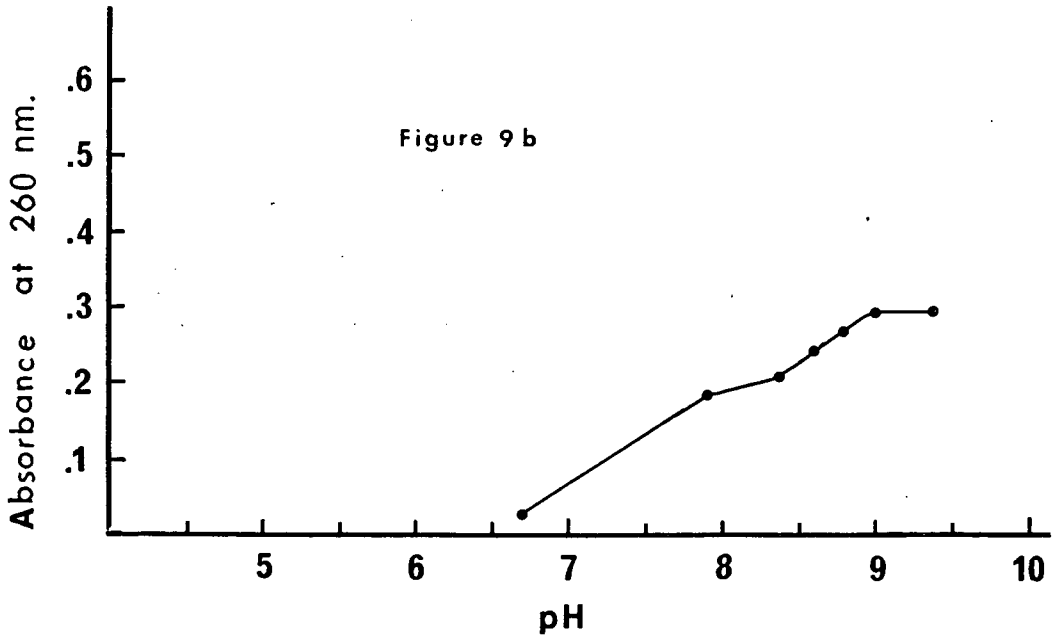

FIGURE 9b. Effect of pH on DEAE-cellulose wash-through RNase activity.

Aliquots (0.2 ml) from fraction number 4 in Figure 8 were assayed at various pH in .05 M Tris-HCl buffer. Incubation was for 60 minutes.

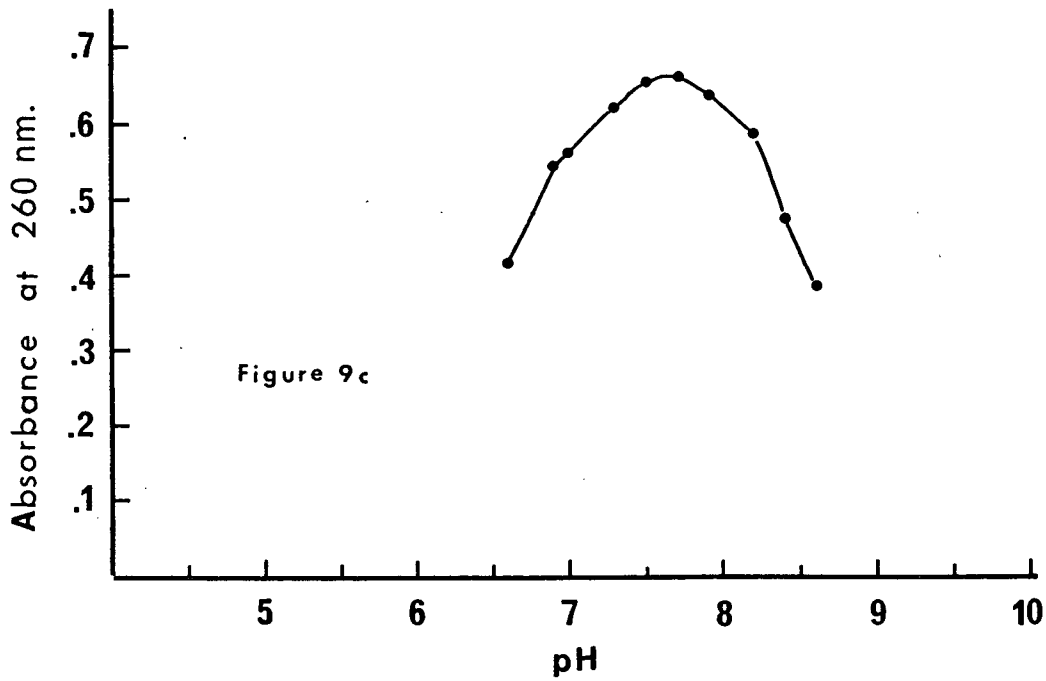

FIGURE 9c.Effect of pH on DEAE-cellulose eluate pH 7.8 RNase activity.

Free pH 7.8 RNase activity eluted between 0.1 and 0.2 M NaCl (eluate fractions 43-65 in Figure 8) were pooled and stored undialyzed for one month at 4° C prior to assay. Aliquots of 0.2 ml were assayed at various pH in .05 M Tris-HCl buffer. Incubation was for 60 minutes.

3.22 Effect of NaCl

The effect of NaCl and buffer concentration on the DEAE-cellulose eluate acid RNase activity was not determined.

DEAE-cellulose eluate pH 9.5 RNase activity was markedly inhibited by NaCl as well as by increasing buffer concentration. The inhibitory effect of NaCl was much greater than could be accounted for in terms of an equivalent increase in ionic strength.

DEAE-cellulose eluate pH 7.8 RNase activity was stimulated by NaCl with maximal activity occurring at the NaCl concentration of 135 mM. NaCl concentrations greater than 180 mM were inhibitory. The stimulatory effect of NaCl on pH 7.8 RNase activity can probably be completely accounted for in terms of ionic strength since the increment in enzyme activity is nearly the same for both a 50 mM increase in NaCl and a 50 mM increase in buffer concentration.

3.23 Effect of $MgCl_2$

Divalent cations were found to strongly inhibit the activity of all three RNases. pH 6.7 and pH 9.4 RNase activities were completely inhibited and pH 7.8 RNase half inhibited at a $MgCl_2$ concentration of 5 mM. However, within a very narrow concentration range around 1 mM, Mg^{++}

TABLE VI. EFFECT OF VARIOUS REAGENTS UPON DEAE-CELLULOSE ELUATE RNase ACTIVITIES

Reagent Added	Final Concentration (mM)	% of Control RNase Activity Assayed in 50 mM Tris-HCl buffer		
		pH 6.7 RNase	pH 9.5 RNase	pH 7.8 RNase
Tris-HCl	25		124	64
	50	100	100	100
	100		66	135
NaCl	5		68	
	10		45	
	25		24	118
	40			129
	100			149
	135			186
	180			164
	225			123
	270			75
	315			46
$MgCl_2$	0.5		16	
	1.0	23		
	2.5	7	9	
	5.0	1	4	55
	10.0		2	40
	15.0			29
	25.0			15
EDTA	0.5		13	
	1.0	123		105
	2.0	88		
	2.5		13	
	10.0			112
pCMB	.05	84		
	.1	72		
	.2	61	36	98
	.3			104
Dithiothreitol	1.0	120		
	1.25			
	2.5		187	95
	5.0	120	190	
	10.0		181	90
	15.0			87

was found to stimulate pH 9.5 RNase activity (see section 3.24).

The influence of $MgCl_2$ on the reaction is likely to be complex. Mg++ may act by directly altering the conformation of the enzyme molecules or by forming a cation bridge between enzyme and other proteins, or between proteins and RNA. It is known[301-303] that divalent cations can considerably alter the structure of RNA molecules and thus affect the accessibility of substrate bonds to the action of RNases.[304] Thus, the mechanism of Mg++ inhibition of RNase activity may be related to the fact that Mg^{++} stabilizes the secondary structure of RNA and may thereby maintain a substrate configuration unfavorable to enzymatic attack.

3.24 Effect of EDTA

One mM EDTA activated acid RNase activity assayed at pH 6.7 by 23%, whereas at a concentration of 2 mM this reagent was slightly inhibitory.

As little as 0.5 mM EDTA produced near complete suppression of pH 9.5 RNase activity. This effect could be reversed by restoring $MgCl_2$ to the reaction mixture at a suitable concentration. $MgCl_2$ was then found to stimulate this enzyme activity by up to 40% (see Fig. 10). This enzyme thus appears to have a divalent cation requirement

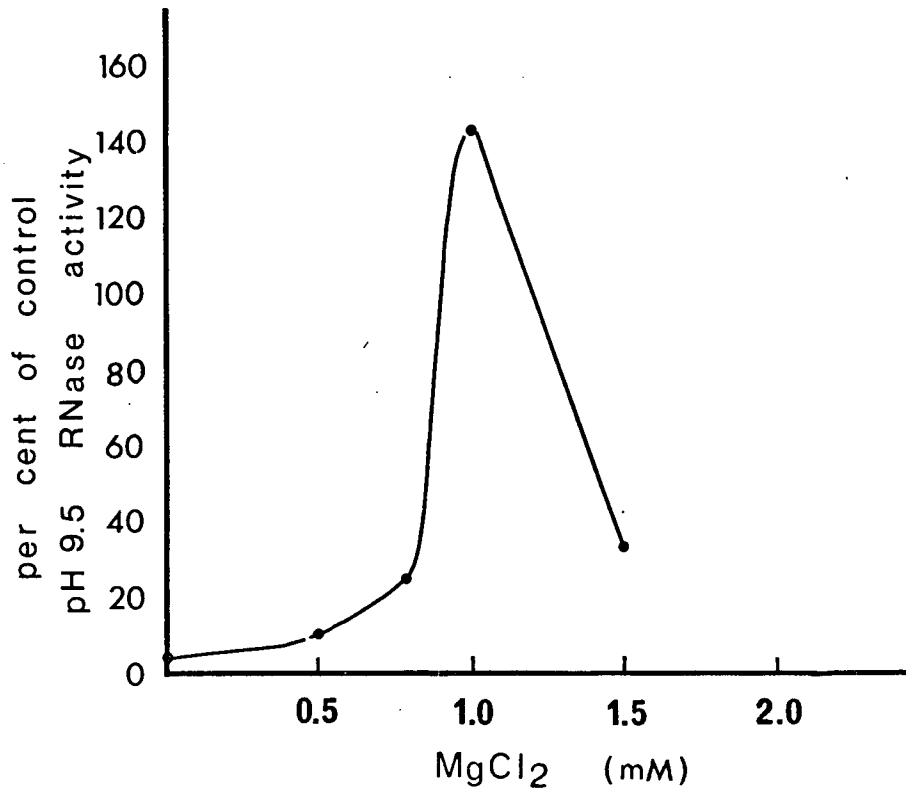

FIGURE 10. Reactivation of EDTA-inhibited pH 9.5 RNase activity by Mg++.

A DEAE-cellulose eluate fraction containing peak pH 9.5 RNase activity (fraction number 5 in Figure 8) was assayed in 50 mM Tris-HCl, pH 9.5 buffer. Various concentrations of MgCl2 were added to incubation mixtures containing 1.0 mM EDTA and the enzyme fraction. Reagents were added to the incubation mixture in the following order: buffer, EDTA, enzyme fraction, MgCl2, sRNA. Incubation was for 60 minutes. Control pH 9.5 RNase activity was that assayed in the absence of both EDTA and MgCl2.

which can be met by Mg++. Hence, the previously observed inhibition by Mg++ of pH 9.5 RNase activity assayed without EDTA was due to the fact that this requirement was already met by traces of divalent cations present in the reaction mixture. The addition of $MgCl_2$ produced super-optimal concentrations of divalent cation which resulted in strong inhibition.

EDTA has no significant effect on free pH 7.8 RNase activity. This supports the conclusion that the inhibitory effect of EDTA on RNase activity in crude enzyme extracts assayed at pH 7.8 is due to the stabilizing influence of this chelating agent on RNase inhibitor activity.

3.25 Effect of pCMB

Parachloromercuribenzoate inhibited acid RNase activity about 40% and pH 9.5 RNase activity about 64% at 0.2 mM concentration.

Free pH 7.8 RNase activity was unaffected by up to 0.3 mM pCMB.

3.26 Effect of ß-mercaptoethanol and dithiothreitol

Dithiothreitol stimulated acid RNase activity 20% at 1 mM. Higher concentrations of either of these sulfhydryl reducing agents had no additional effect. The opposite effects of pCMB and DTT on acid RNase activity are consistent

with the conclusion that this enzyme contains free sulfhydryl group(s) which are required for optimal enzymatic activity.

A similar conclusion can be made with regard to the pH 9.5 RNase activity. The more striking effects of equal concentrations of these reagents on the later enzyme indicates that the critical sulfhydryl groups involved are either more reactive and/or more essential to the activity of this enzyme in comparison with acid RNase.

Dithiothreitol has no significant effect on pH 7.8 RNase activity. This is consistent with its insensitivity to pCMB and suggests that this enzyme has no free sulfhydryl group requirements or that its sulfhydryl groups are unreactive and inaccessible to these reagents at pH 7.8. The lack of influence of DTT, pCMB and EDTA on partially purified pH 7.8 RNase indicates that the previously observed effect of these reagents on RNase activity assayed in crude enzyme preparations at pH 7.8 was mediated through the action of these reagents on RNase inhibitor.

3.27 Effect of detergents

All three RNase activities were almost completely inhibited by the inclusion of .005% (w/v) sodium lauryl sulphate in the assay mixtures (Table VII).

TABLE VII. EFFECT OF VARIOUS DETERGENTS UPON DEAE-CELLULOSE ELUATE RNase ACTIVITIES

Detergent Added	Final Concentration (%)	% of Control RNase Activity assayed in 50 mM Tris-HCl buffer		
		pH 6.7 RNase	pH 9.5 RNase	pH 7.8 RNase
Sodium lauryl sulphate[1]	.005	15	12	0
	.025	18	4	0
Sodium desoxycholate[1]	.05	133	24	107
	.10	43	11	113
Triton X100[2]	.025	171	106	108
	.10	153	95	108

[1]Final concentration given in per cent (weight/volume).

[2]Final concentration given in per cent (volume/volume).

Sodium deoxycholate at a final concentration of .05% (w/v) inhibited pH 9.5 RNase activity by 76% but stimulated pH 6.7 RNase activity by 33%. Increasing the sodium deoxycholate concentration to 0.1% resulted in 57% inhibition of pH 6.7 RNase activity. At these concentrations sodium deoxycholate had no significant effect on pH 7.8 RNase activity.

Both pH 7.8 RNase and pH 9.5 RNase activity was unaffected by 0.1% (v/v) Triton X100, whereas pH 6.7 RNase activity was stimulated 71% and 53% by Triton X100 at concentrations of .025% (v/v) and 0.1% (v/v) respectively.

3.28 Effect of storage

Although crude enzyme preparations routinely stored at 0° for up to two weeks exhibited no noticeable loss in acid RNase activity, at all subsequent stages of purification this enzyme was markedly more labile than pH 7.8 RNase activity. The lability of pH 6.7 RNase activity from brain is consistent with observations of this enzyme activity in other organs by several investigators[190,191,202] who found it to be relatively more heat- and acid-labile than pH 7.8 RNase. In the present study, DEAE-cellulose eluate fractions containing peak pH 6.7 RNase activity lost 40% of their initial activity after storage at 0° for 11 days in 20 mM Tris-HCl, pH 7.4 buffer containing 10% (v/v) glycerol. However, storage at 0° for 13 days in either 20 mM NH_4-acetate, pH 5 buffer or in 20 mM Tris-HCl, pH 6.7 buffer containing 30% (v/v) glycerol resulted in only 8% loss in activity. Stabilization of this enzyme activity could thus be achieved by storage at acid pH or in the presence of 30% (v/v) glycerol.

Partially purified pH 9.5 RNase activity was also found to undergo rapid inactivation upon storage. This inactivation appears to be due to the oxidation of free sulfhydryl groups since β-mercaptoethanol or dithiothreitol were able to reactivate this enzyme and prevent its loss of activity. DEAE-cellulose wash-through pH 9.5 RNase activity

stored at 4° for 14 days in 20 mM Tris-HCl at pH 6.4, 7.0, 8.0 and 9.5 retained 94%, 93%, 68% and 35% respectively of its initial activity. Stored under the same conditions at pH 9.5 in the presence of 5mM ß-merceptoethanol 66% of the original activity was retained.

The pH 7.8 RNase activity was found to be comparatively stable and no attempt was made to determine optimal stabilization conditions for this enzyme.

3.3 DNase and Phosphodiesterase Activities of DEAE-cellulose eluate enzyme fractions

The possibility that the identified RNA-depolymerizing enzyme activities might be due to nucleases or phosphodiesterases capable of cleaving phosphodiester bonds in ribonucleic acid molecules was tested by assaying for these activities using double-stranded calf thymus DNA or bis-p-nitrophenyl phosphate as substrates.

Table VIII shows that the DEAE-cellulose eluate enzyme fractions do not significantly degrade double-stranded DNA but exhibit preferential specificity for the polynucleotide substrate containing ribose and uracil moieties.

Table IX shows that the partially purified enzyme preparations exhibit no significant amounts of either 3'PO4- or 5' PO4-forming phosphodiesterase activity as compared to crude enzyme preparations of whole brain.

TABLE VIII. DNase ACTIVITY OF DEAE-CELLULOSE ELUATE ENZYME FRACTIONS

Enzyme Preparation	Buffer final concentration	Substrate*	Increase in A260 per 60 minute incubation
75-100% saturated $(NH_4)_2SO_4$ precipitable fraction	50 mM Tris-HCl, pH 6.7	sRNA	.815
		DNA	.036
DEAE-cellulose eluate fraction number 32 (Fig. 7)	50 mM Tris-HCl, pH 6.7	sRNA	.234
		DNA	.025
DEAE-cellulose pooled eluate fractions 55-70 (Fig.8)	50 mM Tris-HCl, pH 7.8	sRNA	.860
		DNA	.010
DEAE-cellulose pooled eluate fractions 5-16 (Fig. 8)	50 mM Tris-HCl, pH 9.5	sRNA	.301
		DNA	.011

*0.5 ml of a 0.1% (w/v) stock solution of either sRNA dissolved in distilled H_2O or double-stranded calf thymus DNA dissolved in 10 mM NaCl was added to the incubation mixture as substrate. Other details of assay procedure are described in section 2.142 of Methods.

TABLE IX. PHOSPHODIESTERASE ACTIVITY OF DEAE-CELLULOSE ELUATE ENZYME FRACTIONS*

Enzyme Preparation	Final buffer concentration	Increase in A400 per 60 minute incubation
0.5 ml whole brain homogenate	33 mM Tris-HCl, pH 8.9	1.363
	33 mM NH_4-acetate, pH 5.0	1.589
0.5 ml whole brain extract	33 mM Tris-HCl, pH 8.9	0.270
.05 ml 75-100% saturated $(NH_4)_2SO_4$ fraction	33 mM Tris-HCl, pH 6.7	.047
0.2 ml DEAE-cellulose eluate fraction #32 (Fig. 7)	33 mM Tris-HCl, pH 6.7	.000
0.2 ml DEAE-cellulose eluate pooled fractions 55-70 (Fig.8)	33 mM Tris-HCl, pH 7.8	.017
0.2 ml DEAE-cellulose eluate pooled fractions 5-16 (Fig. 8)	33 mM Tris-HCl, pH 9.5	.000

*Conditions of assay are as described in section 2.143 of Methods.

3.4 Intracellular Distribution of RNase Activities and RNase Inhibitor Activity

Isotonic sucrose homogenates of adult rat whole brain were prepared and fractionated by differential centrifugation into nuclear, crude mitochondrial, microsomal, and cytosol fractions according to the procedure described in section 2.12 of Methods. Each subcellular fraction was assayed for RNase activity in 50 mM Tris-HCl buffer at pH 6.7, pH 9.5, and at pH 7.8 with and without 0.2 mM pCMB.

The results recorded in Table X , XI, and XII show that for each RNase the sum of the separate activities of each subcellular fraction is significantly greater than the total activity expressed in the initial isotonic sucrose homogenate. Total recovery of each RNase activity in all the subcellular fractions assayed at pH 6.7, pH 9.5 and pH 7.8 was 192%, 167% and 205% respectively of the activity expressed in the isotonic sucrose whole homogenate. This activation of RNase activities is due to the rehomogenization of the separated subcellular particulate fractions. This procedure results in the liberation of considerable RNase activity which was initially present in the isotonic sucrose whole homogenate in a latent, non-functional form either bound to or compartmentalized within organelles. The total recovery of each RNase activity in all the subcellular fractions assayed at pH 6.7, pH 9.5, and pH 7.8 was, however, only 56%,

63%, and 76% respectively of the activity expressed in 0.1% Triton X100 homogenates. This indicates that homogenization of the separated subcellular particulate fractions in isotonic sucrose does not liberate all of the total detergent-extractable RNase activity.

Table X shows that the pH 6.7 RNase activity which is expressed under these conditions is predominantly localized in the crude mitochondrial and cytosol fractions. Of the total recovered pH 6.7 RNase activity, 41% was present in the crude mitochondrial fraction and 31% in the cytosol fraction. The specific activity of this enzyme in the cytosol fraction was three-fold greater than that of any of the other subcellular fractions.

Studies[295,305] of the intracellular distribution of acid RNase in rat liver by differential and sucrose density gradient centrifugation have concluded that the acid RNase activity associated with the crude mitochondrial fraction is localized in lysosomes. In view of the similarities of the pH 6.7 RNase activity reported in the present study to that of acid RNase of rat liver, it seems likely that the large amount of pH 6.7 RNase activity of rat brain which is recovered in the crude mitochondrial fraction may also be localized within the lysosomes of this fraction.

TABLE X. INTRACELLULAR DISTRIBUTION OF pH 6.7 RNase ACTIVITY

Enzyme Preparation	pH 6.7 RNase Activity				
	Total Activity (units)	Specific activity (units/mg protein)	% of sucrose homogenate activity recovered	% of the sum of activities recovered in all the subcellular fractions	Total activity (units) recovered per gram wet weight of whole brain
0.1% Triton X100 homogenate	420.0	0.55	347	181	70.0
Isotonic sucrose homogenate	120.9	0.16	100	52	20.2
800 x g pellet (nuclear fraction)	40.0	0.23	33	17	6.6
8,000 x g pellet (crude mitochondrial fraction)	94.8	0.29	78	41	15.8
105,000 x g pellet (microsomal fraction)	23.8	0.21	20	10	4.0
105,000 x g supernatant (cytosol fraction)	73.2	0.62	61	32	12.2

In the experiments recorded in the above and following Tables, 60 mls of a 10% (wet wt. brain/final volume) isotonic sucrose homogenate of adult (4-month-old) rat whole brains was fractionated according to the procedure described in section 2.12 of Methods. Pelleted subcellular fractions were resuspended by homogenization in 0.32 M sucrose, brought to final volume with 0.32 M sucrose, and aliquots of each fraction were assayed in 50 mM Tris-HCl buffer at the appropriate pH.

Although the studies on rat liver found that little acid RNase was present in the cytosol fraction, De Lamirande and Allard[202] found that acid RNase of intestinal mucosa and kidney, in contrast with liver, was present mainly in the soluble fraction. It is not known to what extent such apparent tissue-specific differences in the intracellular distribution of this RNase activity may represent differences in the fragility of lysosomes or other subcellular particles and, hence, in the release of enzyme into the soluble fraction during the isolation of subcellular particles. It is also possible that a large portion of this enzyme, at least in brain, may be a normal constituent of the cell sap and the activity found in other subcellular fractions may represent enzyme which has been adsorbed onto sedimentable particles during their isolation. Such adsorption phenomena are a well known[296] source of artifacts in subcellular distribution studies. However, in rat liver, Rahman[201,206] found no evidence of the adsorption of soluble RNases to subcellular particles.

Table XI shows that pH 9.5 RNase activity is also found predominantly in the crude mitochondrial and cytosol fractions. However, proportionally more of this enzyme activity is recovered in the crude mitochondrial fraction (57%) than in the cytosol (20%). Both these fractions have equal specific activities which were twice that of the nuclear and microsomal fractions.

TABLE XI. INTRACELLULAR DISTRIBUTION OF pH 9.5 RNase ACTIVITY

Enzyme Preparation	pH 9.5 RNase Activity: Total activity (units)	Specific activity (units/mg protein)	% of sucrose homogenate activity recovered	% of the sum of activities recovered in all the subcellular fractions	Total activity (units) recovered per gram wet weight of whole brain
0.1% Triton X100 homogenate	244.2	0.32	263	158	40.7
Isotonic sucrose homogenate	93.0	0.12	100	60	15.5
800 x g pellet (nuclear fraction)	21.8	0.13	23	14	3.6
8,000 x g pellet (crude mitochondrial fraction)	93.6	0.29	101	60	15.6
105,000 x g pellet (microsomal fraction)	10.1	0.09	11	7	1.7
105,000 x g supernatant (cytosol fraction)	29.4	0.28	32	19	4.9

The intracellular distribution of the recovered pH 9.5 RNase activity thus resembles that of pH 6.5 RNase except that a larger portion of this activity is obtained in the crude mitochondrial fraction and less in the microsomal and cytosol fractions. This distribution differs from that reported by Rahman[206] for pH 9.5 RNase of rat liver. In liver, relatively more of the total cellular content of pH 9.5 RNase activity was recovered in the microsomal fraction and very little was found in the cytosol fraction.

Table XII shows that the largest amount 63% of recovered free pH 7.8 RNase activity and the highest specific activity of this enzyme was found in the crude mitochondrial fraction.

However, assuming that 56% of the optimal activity of pH 9.5 RNase is retained in assays at pH 7.8, 8.7 units (per gram wet weight) of the total RNase activity expressed in the crude mitochondrial fraction assayed at pH 7.8 (13.2 units/g wet weight) can be accounted for in terms of pH 9.5 RNase activity expressed at pH 7.8. This leaves a remainder of 4.5 units((per gram wet weight) which must represent the actual activity contributed by free pH 7.8 RNase.

It is unlikely that in the intact cell the soluble cell sap contains any free pH 7.8 RNase activity in view of the large excess of free RNase inhibitor found in the cytosol.

TABLE XII. INTRACELLULAR DISTRIBUTION OF FREE pH 7.8 RNase ACTIVITY

Enzyme Preparation	Free pH 7.8 RNase Activity				
	Total activity (units)	Specific activity (units/mg protein)	% of sucrose homogenate activity recovered	% of the sum of activities recovered in all the subcellular fractions	Total activity (units) recovered per gram wet weight of whole brain
0.1% Triton X100 homogenate	163.2	0.21	268	131	27.2
Isotonic sucrose homogenate	60.9	0.08	100	49	10.2
800 x g pellet (nuclear fraction)	24.4	0.15	40	20	4.0
8,000 x g pellet (crude mitochondrial fraction)	78.9	0.24	130	63	13.2
105,000 x g pellet (microsomal fraction)	10.6	0.10	17	8	1.8
105,000 x g supernatant (cytosol fraction)	11.0	0.09	18	9	1.8

The 9% of free RNase activity assayed at pH 7.8 which is recovered in this fraction can be completely accounted for in terms of pH 9.5 RNase activity expressed at pH 7.8.

Table XIII shows that total pH 7.8 RNase activity assayed in the presence of 0.2 mM pCMB was mostly recovered in the cytosol fraction, and the specific activity of this fraction was 5- to 10-fold greater than that of the other subcellular fractions. From the difference between free and total pH 7.8 RNase activity it can be calculated that an apparent 88% of the latent, inhibitor-bound pH 7.8 RNase is present in the cytosol (Table XIV).

The pH 7.8 RNase activity in the crude mitochondrial fraction assayed in the presence of 0.2 mM pCMB was 38% lower than that assayed without pCMB, indicating that pCMB has a net inhibitory effect on pH 7.8 RNase activity in this fraction (see Fig. 12a). From the failure of pCMB to stimulate RNase activity assayed at pH 7.8 in the crude mitochondrial fraction, it would appear that this fraction contains no inhibitor-bound pH 7.8 RNase despite the fact that this fraction contains 63% of the total recovered free pH 7.8 RNase activity and 27% of the total recovered free RNase inhibitor activity (Table XV).

TABLE XIII. INTRACELLULAR DISTRIBUTION OF TOTAL pH 7.8 RNase ACTIVITY

Enzyme Preparation	Total pH 7.8 RNase Activity				
	Total activity (units)	Specific activity (units/mg protein)	% of sucrose homogenate activity recovered	% of the sum of activities recovered in all the sub-cellular fractions	Total activity (units) recovered per gram wet weight of whole brain
0.1% Triton X100 homogenate	244.2	0.32	127	107	40.7
Isotonic sucrose homogenate	192.6	0.25	100	85	32.1
800 x g pellet (nuclear fraction)	38.0	0.22	20	17	6.3
8,000 x g pellet (crude mitochondrial fraction)	49.0	0.15	25	22	8.2
105,000 x g pellet (microsomal fraction)	12.5	0.11	6	5	2.1
105,000 x g supernatant (cytosol fraction)	128.2	1.08	67	56	21.4

TABLE XIV. INTRACELLULAR DISTRIBUTION OF LATENT pH 7.8 RNase ACTIVITY

Enzyme Preparation	Latent pH 7.8 RNase Activity*				
	Total activity (units)	Specific activity (units/mg protein)	% of sucrose homogenate activity recovered	% of the sum of activities recovered in all the sub-cellular fractions	Total activity (units) recovered per gram wet weight of whole brain
0.1% Triton X100 homogenate	81.0	0.11	62	61	13.5
Isotonic sucrose homogenate	131.7	0.17	100	99	21.9
800 x g pellet (nuclear fraction)	13.6	0.08	10	10	2.3
8,000 x g pellet (crude mitochondrial fraction)	-29.9[a]		0	0	0
105,000 x g pellet (microsomal fraction)	1.9	0.02	1	1	0.3
105,000 x g supernatant (cytosol fraction)	117.2	0.98	89	88	19.5

*Latent or inhibitor-bound pH 7.8 RNase activity is defined as the activity released upon treatment with pCMB, and was calculated by subtracting the free pH 7.8 RNase activity (Table XII) from the total pH 7.8 RNase activity assayed in the presence of 0.2 mM pCMB (Table XIII).

[a]This negative value reflects the net inhibitory effect of pCMB on RNase activity assayed at pH 7.8 in the crude mitochondrial fraction. This value was taken as zero in calculating total recovery.

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TABLE XV. INTRACELLULAR DISTRIBUTION OF FREE RNase INHIBITOR ACTIVITY

Enzyme Preparation	Free RNase Inhibitor Activity* Total Activity (units)	Specific Activity (units/mg protein)	% of total protein recovered in fraction	% of sucrose homogenate activity recovered	% of sum of activities recovered in all sub-cellular fractions	Total activity (units recovered per gram wet weight of whole brain
Isotonic sucrose homogenate	6,660	8.54	100	100	100	1,110.0
800 x g pellet (nuclear fraction)	217.8	1.26	3	22	3	36.3
8,000 x g pellet (crude mitochondrial fraction)	1,800.0	5.56	27	42	27	300.0
105,000 x g pellet (microsomal fraction)	315.0	2.84	5	14	5	52.5
105,000 x g supernatant (cytosol fraction)	4,317.5	36.34	65	15	65	719.6

*Free RNase inhibitor activity was assayed according to the procedure described in section 2.144 of Methods. The incubation mixture contained 0.5 ng. bovine pancreatic RNase A, 1 mM EDTA, and various aliquots of tissue fractions suitably diluted with ice-cold .02 M Tris-HCl, pH 7.8 buffer so as to yield inhibitions of control bovine pancreatic RNase A activity between 0 and 80%. Incubation was for 30 minutes. A unit of free inhibitor activity is defined as the amount of inhibitor which produces 50% inhibition of the activity of 0.5 ng. bovine pancreatic RNase A. Units of inhibitor activity were interpolated from standard curves expressing per cent inhibition of the control activity of 0.5 ng pancreatic RNase A as a function of the amount of tissue sample added.

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It is unlikely that these results can be explained in terms of differential compartmentalization of the free pH 7.8 RNase activity and the free RNase inhibitor activity since the rehomogenization to which this fraction was subjected and the hypotonic condition of assay would be expected to largely remove the constraints to interaction between segregated molecular species.

The data suggests that the crude mitochondrial fraction contains an alkaline RNase differing from that of the cytosol in being (1) inhibited by pCMB, and (2) insensitive to inhibition by the free RNase inhibitor endogenous to the crude mitochondrial fraction.

In rat liver, both Roth[195] and Shortman[213] found the mitochondrial pH 7.8 RNase activity to be sensitive to inhibition by the free RNase inhibitor present in the cytosol. Roth[187] inferred that the predominant fraction of mitochondrial pH 7.8 RNase activity must hence be present in the free form. He also reported[190] evidence for the presence of some inhibitor-bound pH 7.8 RNase in the crude mitochondrial fraction of rat liver.

The simplest explanation for the apparent absence of detectable inhibitor-bound pH 7.8 RNase in the crude mitochondrial fraction of brain lies in the fact that inhibitor-bound pH 7.8 RNase is normally indirectly inferred from the

activating effect of pCMB; but due to the high pH 9.5 RNase content of the crude mitochondrial fraction, pCMB activation of inhibitor-bound pH 7.8 RNase is more than counterbalanced by the inhibitory effect of pCMB on the residual pH 9.5 RNase activity expressed in assays at pH 7.8.

The results thus represent a composite effect reflecting the inability of the assay conditions to clearly discriminate between the RNase activities present in the crude mitochondrial fraction. The RNase activity measured at a given pH is not due solely to a single enzyme but is the result of the combined actions of the multiple enzyme species present in this subcellular fraction.

Table XV shows that free RNase inhibitor activity assayed as described in section 2.144 of Methods was found predominantly in the cytosol (66% of total recovered activity) and crude mitochondrial fraction (27% of total recovered activity). The small amount of inhibitor activity recovered in the nuclear fraction probably represents the degree to which this fraction is contaminated with adhering cytoplasmic material.

The intracellular distribution of free RNase inhibitor activity in brain closely resembles that reported for rat liver. Roth[187] found RNase inhibitor activity to be high in the cytosol and low in the microsomal and nuclear fractions

of rat liver. Roth[219] has more recently reported the absence of any detectable free RNase inhibitor in purified nuclei from rat liver.

It is of special interest that the sum of the free RNase inhibitor activities of the subcellular fractions was found to be equal to the total free RNase activity in the isotonic sucrose whole homogenate. This component of the RNase enzyme system thus exhibits no subcellular structure-linked latency and this may be critical to the functioning of this protein in titrating the level of activity of pH 7.8 RNase in the intact cell.

3.5 Characterization of RNase activities in separated subcellular fractions

In view of the inability of the assay conditions used to clearly differentiate the specific contribution of each of the multiple enzyme species to the RNase activity measured at a given pH, a more reliable determination of the activity contributed by each component RNase seemed to require their more thorough characterization and separation starting from a particular subcellular fraction. Also, in view of the rather unremarkable similarities of the three RNases detected in brain to those previously reported in rat liver, a more detailed study of the RNase activities of the individual subcellular fractions was made in an attempt to detect novel RNase activities or distinctive characteristics not detectable

in previous studies of whole homogenates and extracts.

The RNase activity of each subcellular fraction, isolated from isotonic sucrose homogenates of adult rat brain, was assayed over a range of hydrogen ion concentrations with and without 1 mM EDTA, 0.2 mM pCMB or 2.0 M urea. The control pH curves of all four subcellular fractions in the absence of any added reagents exhibited higher activity in the acid pH region than in the alkaline region. The control pH optimum for acid RNase activity was pH 6.7 in Tris-HCl buffer and pH 6.3 in NH_4 acetate buffer as for the whole cell homogenate. However, each subcellular fraction differed from the whole homogenate in the alkaline region of the control activity versus pH profile.

The control pH curve of the nuclear fraction has a bimodal appearance suggestive of activity maximae at pH 8 and 8.6. The crude mitochondrial fraction exhibits a broad plateau of activity between pH 8 and 9.5, and the microsomal fraction has a distinct activity maxima at pH 8.9. The pH curve of freshly prepared cytosol fraction exhibits a deep trough between pH 7 and 8.5 with activity minima occurring at pH 7.5. Above pH 8.5 the cytosol fraction exhibits a broad activity plateau with no distinct maxima (see Fig. 14 on page 138).

The effect of 0.2 mM pCMB on the RNase activities of the nuclear and mitochondrial fractions is shown in Figures 11a and 12a respectively. This sulfhydryl blocking agent stimulated activity in the nuclear fraction between pH 7 and 8.5 with a maximal stimulation of 53% occurring at the pH minima of control activity. A slight inhibition of activity was observed above pH 8.6 and below pH 6.7. The mitochondrial fraction RNase activity, however, showed a consistent inhibition of about 40% by 0.2 mM pCMB throughout the entire pH range tested. The magnitude of the measured activity is the net effect resulting from differential activation and inhibition by pCMB of the various enzyme species present in this fraction, and is interpreted to indicate that the pH 9.5 RNase/inhibitor-bound pH 7.8 RNase ratio for this fraction is considerably higher than that for nuclear and cytosol fractions. More direct verification of this putative explanation will require the separation of these enzyme components from each other starting from the crude mitochondrial fraction.

The effect of 1 mM EDTA on RNase activity in nuclear and mitochondrial fractions is shown in Figures 11b and 12b respectively. This concentration of EDTA completely abolishes mitochondrial alkaline RNase activity whereas about 50% of the control alkaline RNase activity is retained in the nuclear fraction.

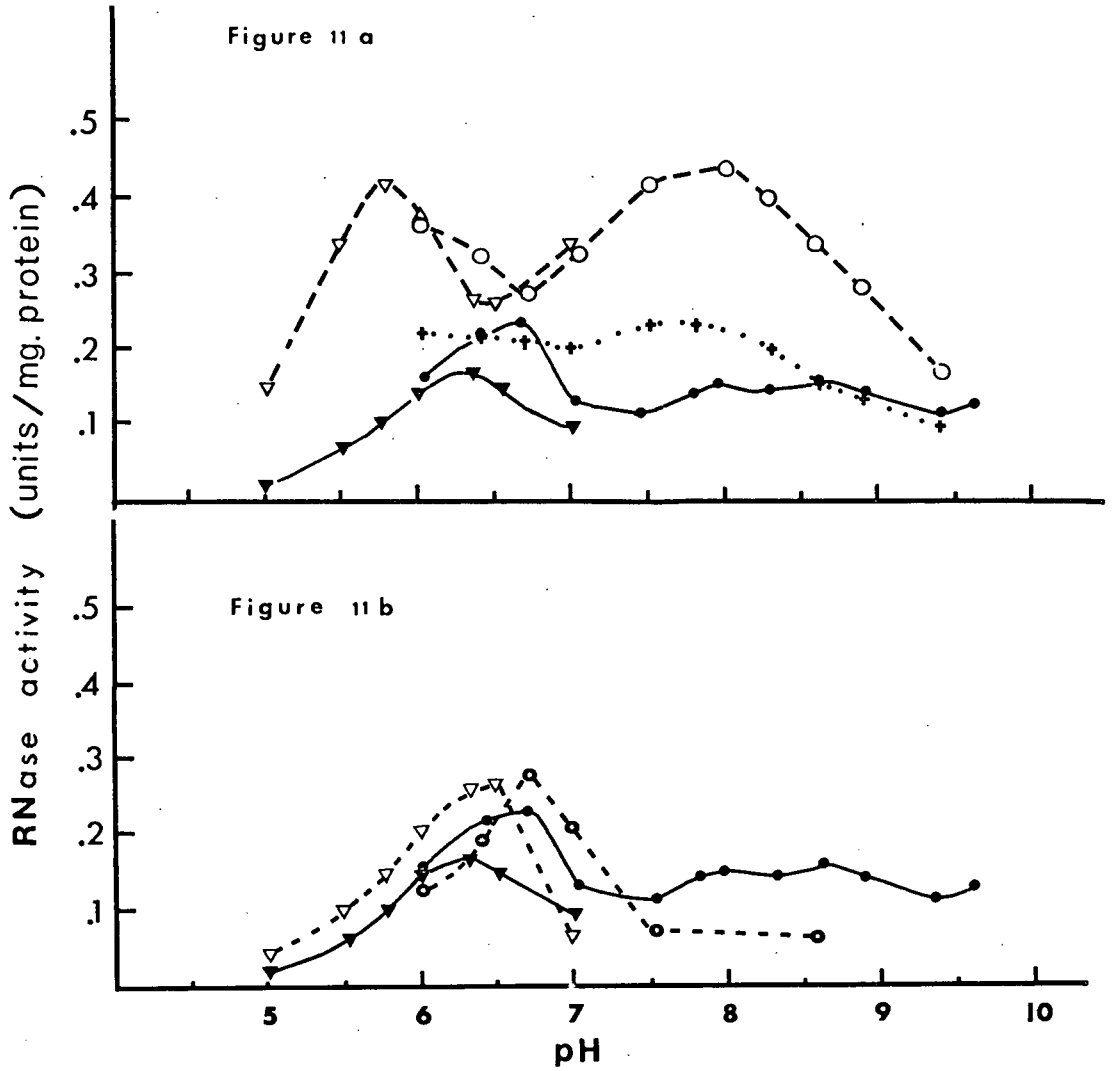

FIGURE 11. The RNase activity in the nuclear fraction isolated from adult rat whole brain (a) in the presence of 2.0 M urea and 0.2 mM pCMB, and (b) in the presence of 1.0 mM EDTA.

Aliquots of 0.2 ml of the nuclear fraction were assayed in 50 mM buffer in the absence of any added reagents (NH4-acetate —▼—; Tris-HCl —●—), and in the presence of either 2.0 M urea (NH4-acetate -▽--; Tris-HCl--○--), or 0.2 mM pCMB (Tris-HCl ··+··), or 1.0 mM EDTA (NH4-acetate --▽--; Tris-HCl --○--). Incubation was for 60 minutes.

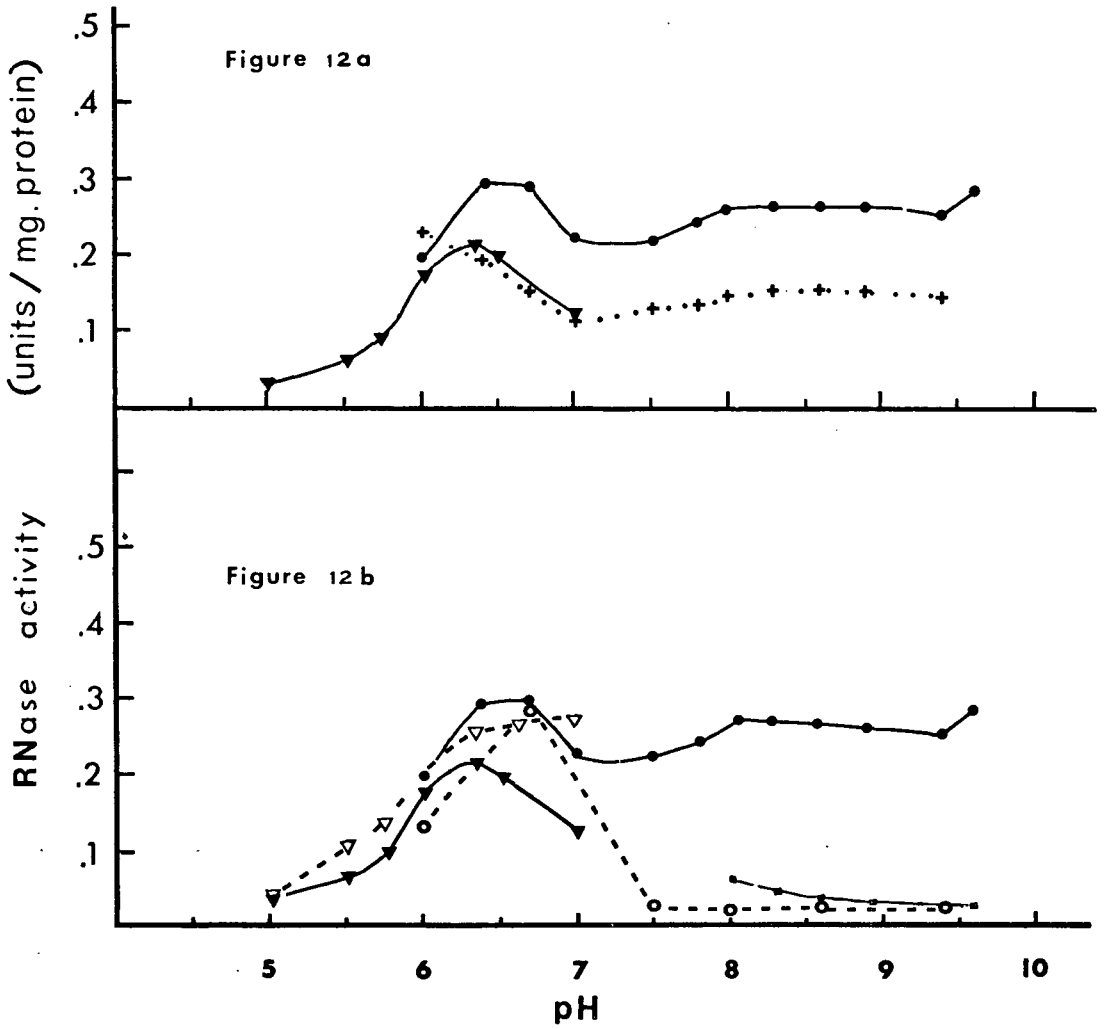

FIGURE 12. The RNase activity in the crude mitochondrial fraction isolated from adult rat whole brain: (a) effect of 0.2 mM pCMB, and (b) effect of 1.0 mM EDTA and 0.5 mM MgCl2.

Aliquots of 0.1 ml of the crude mitochondrial fraction were assayed in 50 mM buffer in the absence of any added reagents (NH4-acetate —▼— ; Tris-HCl —•—) and in the presence of either 0.2 mM pCMB (Tris-HCl ··+··), or 1.0 mM EDTA (NH4-acetate --▽-- ; Tris-HCl --○--), or 0.5 mM MgCl2 (Tris-HCl —▪—). Incubation was for 60 minutes.

In ammonium acetate buffer, both mitochondrial and nuclear acid RNase activity is slightly stimulated by 1 mM EDTA, with maximal stimulation occurring above pH 6.0. In Tris-HCl buffer, however, the acid RNase activity assayed below pH 6.7 is slightly inhibited by 1 mM EDTA in both mitochondrial and nuclear fractions. In Tris-HCl buffer above pH 6.7, acid RNase activity is stimulated in the nuclear fraction and unaffected in the mitochondrial fraction.

The effect of 2.0 M urea on RNase activity in the nuclear and microsomal fractions is shown in Figures 11a and 13 respectively. Acid RNase activity is markedly stimulated 140% and 100% in nuclear and microsomal fractions respectively and the pH optimum of this enzyme activity is shifted to pH 5.8. In the alkaline region of the pH curve, 2.0 M urea stimulates RNase activity maximally (193%) at pH 8 in the nuclear fraction. Urea-activation of RNase activity assayed at this pH cannot be completely accounted for in terms of the release of inhibitor-bound pH 7.8 RNase activity since the level of activity evoked in the presence of urea is 2-fold greater than that obtained in the presence of an optimal concentration (0.2 mM) of pCMB. Hence, the activation by urea and pCMB of RNase activity assayed at pH 7.8 must be effected at least in part by different mechanisms.

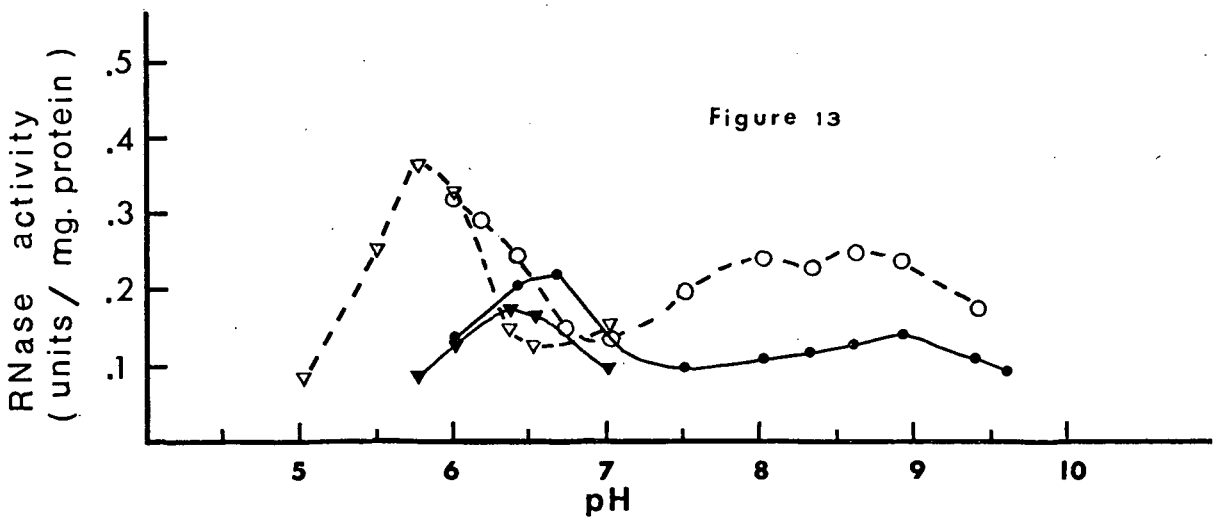

FIGURE 13. Effect of 2.0 M urea on the RNase activity of the microsomal fraction.

Aliquots of 0.2 ml of the microsomal fraction were assayed in the absence of any added reagents (NH4-acetate —▼—; Tris-HCl —●—) and in the presence of 2.0 M urea (NH4-acetate --▽--; Tris-HCl --○--). Incubation was for 60 minutes.

Urea stimulates alkaline RNase activity in the microsomal fraction to a lesser extent (100%) than in the nuclear fraction. In addition to the activity maxima at pH 8, a second activity maxima at pH 8.6 is observed in this fraction. This activity may correspond to the microsome-bound RNase reported by other workers[223-225,286] and known to be activated by urea. Thus, both acid and alkaline RNase activites are markedly stimulated in the presence of 2.0 M urea. The stimulating effect of urea on pH 9.5 RNase is least marked. The optimal concentration of urea was not determined.

The increased yield of acid-soluble oligoribonucleotides in the presence of urea is probably not due to a direct stimulation of the RNases since the direct action of urea on these enzymes would be expected to unfold their structure and inhibit their activity. Rather, the stimulating effect of urea may be due to (1) dissociation of non-specific protein-RNase and protein-RNA complexes and aggregates thereby facilitating enzyme-substrate interaction; (2) disruption of the H-bonded secondary structure of the RNA thereby making the substrate more vulnerable to enzyme action, and/or (3) since RNases may produce single-stranded endonucleolytic breaks in RNA, leaving oligoribonucleotide sequences H-bonded to the intact strand, urea may facilitate

the release of those products of the enzyme action which remain H-bonded. Elucidation of the precise contribution of each of these mechanisms to theobserved composite effect of urea requires further experiments. A similar enhancement of pancreatic RNase A activity by urea has been reported by Kalnitsky et al.[297] These workers found that one-third of the increase could be accounted for by the solubilizing effect of urea on the products of the reaction; i.e., facilitation of the release of H-bonded base pairs into acid-soluble form.

This study of the RNase activities of individiual subcellular fractions suggests the presence of an additional alkaline RNase activity distinct from those previously detected in the whole cell homogenates and extracts. This microsomal alkaline RNase activity with an apparent pH optimum between pH 8.6 and 8.9 was presumably masked by the predominance of pH 7.8 and pH 9.5 RNase activities in assays of whole cell homogenates and extracts.

It may be possible to take advantage of the differential intracellular localization of the various RNases and reduce the complexity of the system under observation by attempting to separate and purify the various enzyme activities present within a particular subcellular fraction, rather than starting from the whole cell homogenate.

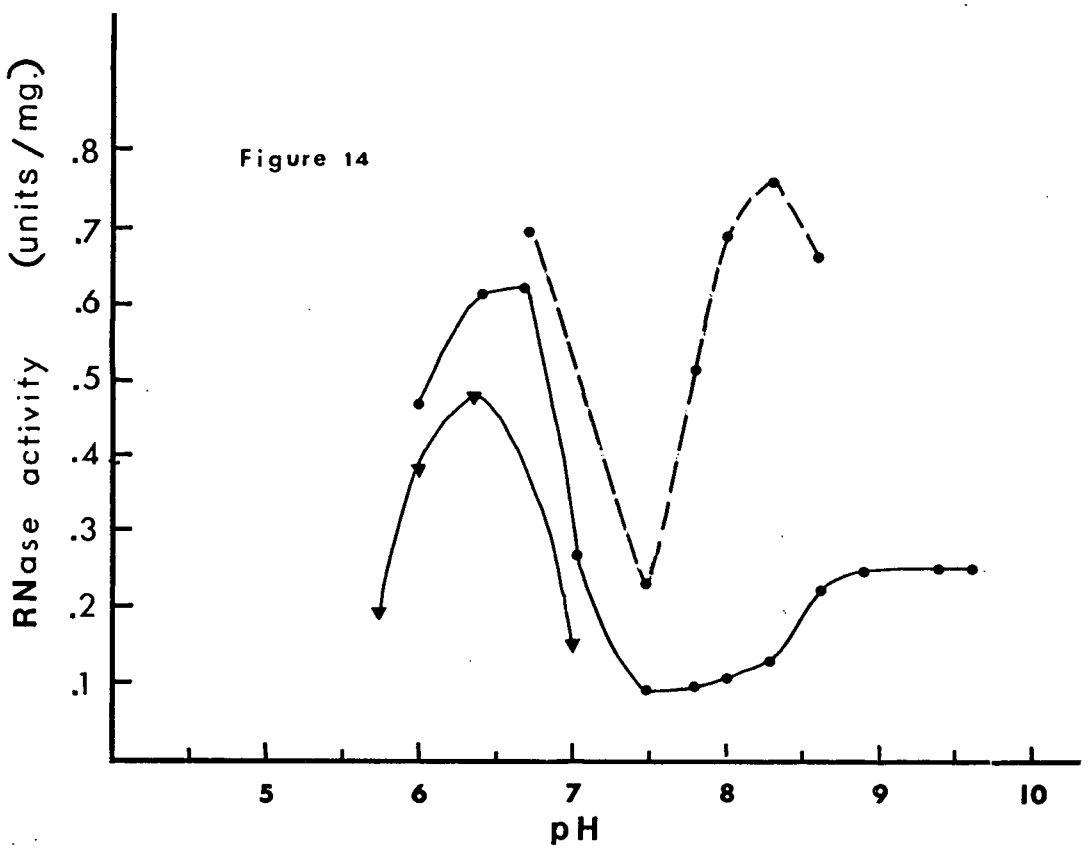

FIGURE 14. RNase activity as a function of pH in freshly prepared and aged cytosol fraction.

Aliquots of 0.2 ml of cytosol were assayed immediately upon preparation (NH4-acetate —▼—; Tris-HCl —•—) and after two weeks storage at 0° (Tris-HCl - -•- -). Incubation was for 60 minutes.

3.6 Developmental Changes in RNase Activities and in RNase Inhibitor Activity in Rat Whole Brain

Figures 15, 16 and 17 show the specific activity levels of RNase assayed at pH 6.7, 9.5 and 7.8 in 0.1% Triton X100 whole brain homogenates of rats at various stages of postnatal maturation.

The specific activity of RNase assayed at pH 6.7 is highest at birth, begins to decline gradually at about day 7, then more rapidly after day 22 until about day 32 after which it remains relatively constant. The specific activity at day 32 is 60% of that at day 1. However, on a per unit wet weight of brain basis the activity progressively increases from birth, peaks at day 22 (120% of day 1 level) and falls rapidly thereafter (1.5-fold decrease) to reach adult levels by day 32 (87% of day 1 level).

Both the specific activity and the activity per gram wet weight brain of RNase assayed at pH 9.5 are minimal at birth, rise rapidly from day 7 to peak at day 22, and subsequently exhibit a gradual but distinct decline throughout adulthood. Specific activities at day 22 and 8 months are 224% and 152% respectively of that at day 1.

The developmental profile of free pH 7.8 RNase activity is similar to that of RNase activity assayed at pH 9.5 except that the lag in the increase up to age 7 days is not observed

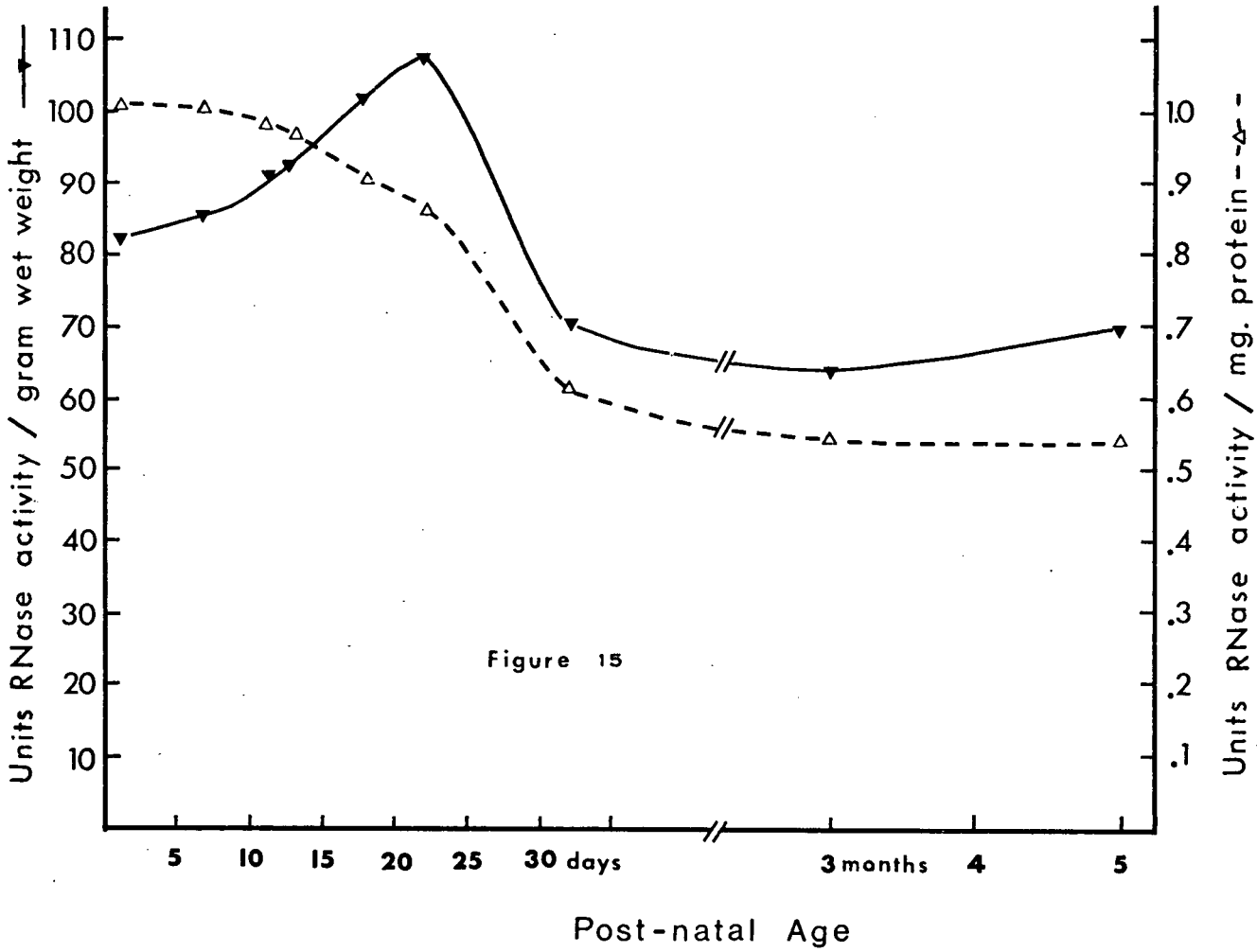

FIGURE 15. Changes in acid RNase activity during postnatal development.

RNase activity in 0.1% Triton X100 homogenates of adult rat whole brain was assayed at pH 6.7 in 50 mM Tris-HCl buffer. Aliquots of 0.05 ml of homogenate were used for assay. Incubation was for 30 minutes.

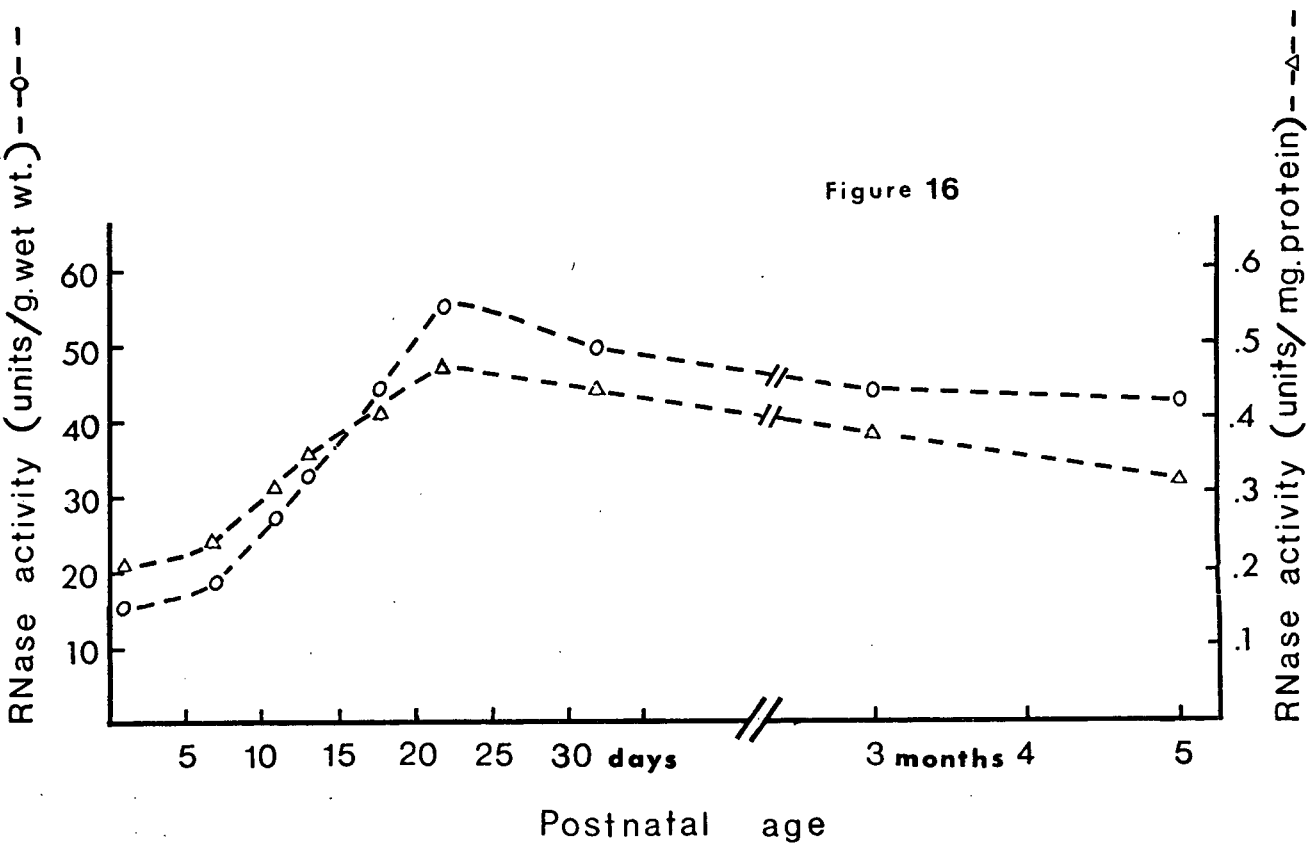

FIGURE 16. Postnatal developmental changes in pH 9.5 RNase activity.

Incubation was for 60 minutes. All other conditions were as in legend to Figure 15, except the assay was conducted at pH 9.5.

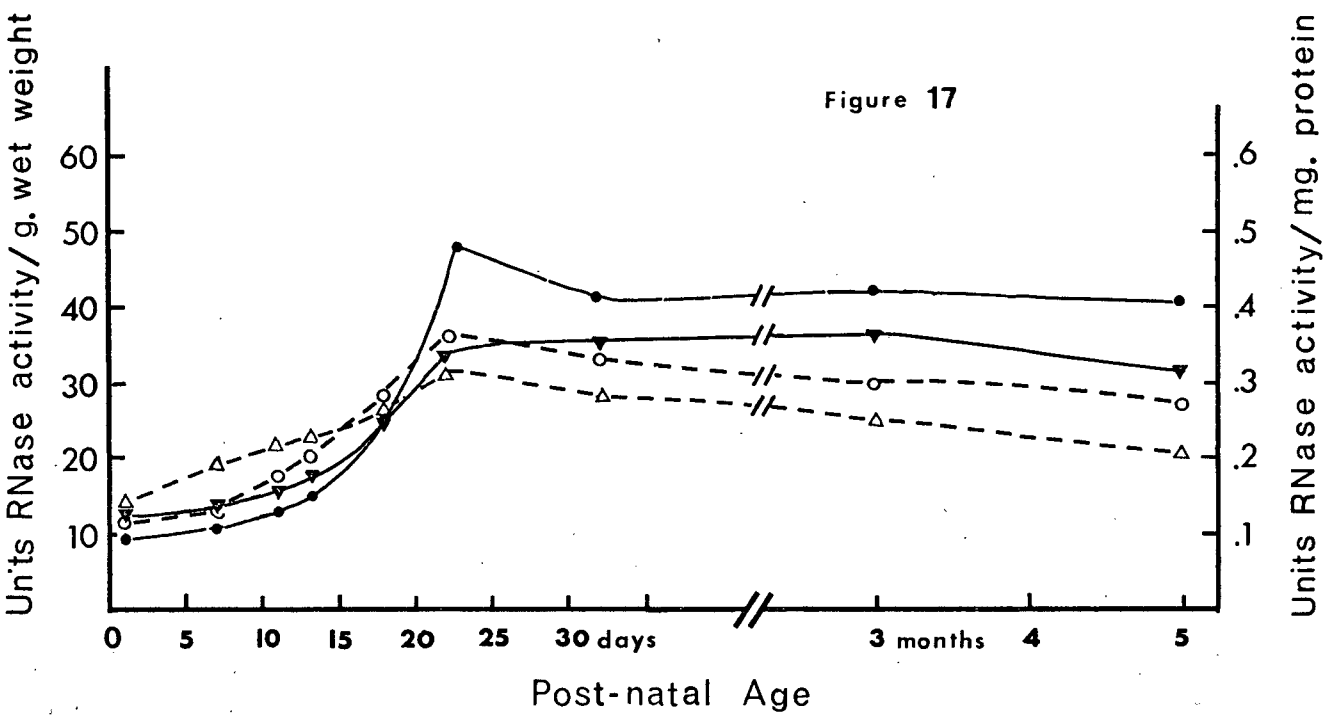

FIGURE 17. Postnatal developmental changes in pH 7.8 RNase activity.

Free pH 7.8 RNase activity assayed in the absence of pCMB expressed in units/mg protein --△-- and in units/gram wet weight brain --o--. Total pH 7.8 RNase activity assayed in the presence of 0.2 mM pCMB expressed in units/mg protein —▼— and in units/gram wet weight brain —●—. Incubation was for 60 minutes. All other conditions were as in legend to Figure 15.

and the rate of increase is more gradual. The magnitude of the net increase, however, is nearly the same. The specific activity observed at age 22 days and 5 months is 221% and 150% respectively of that at day 1. Free pH 7.8 RNase specific activity also undergoes a similar gradual decline throughout adulthood with the specific activity at 5 months being 68% of the peak specific activity at age 22 days.

Total pH 7.8 RNase activity (assayed in the presence of 0.2 mM pCMB) exhibits a rapid 2.3-fold increase in specific activity between day 10 and day 22 after which it remains relatively constant throughout adulthood. Parachloromercuribenzoate is inhibitory at all ages prior to day 20. The shape of the difference curve between the specific activities of free and total pH 7.8 RNase is interpreted as an index of the inhibitor-bound pH 7.8 RNase/pH 9.5 RNase activity ratio. Thus, this ratio is relatively low at birth and decreases to a minimum at 10 days due to a proportionally greater increase in pH 9.5 RNase specific activity during this period. It then increases and attains day 1 values by age 18 days due to a proportionally greater increase during this time in free RNase inhibitor and hence in inhibitor-bound pH 7.8 RNase. The fact that after age 20 days the net effect of pCMB is a stimulation of RNase activity assayed at pH 7.8 indicates that this ratio has become sufficiently large so that the inhibitory

effect of pCMB on pH 9.5 RNase activity expressed in assays at pH 7.8 is masked by the overriding stimulatory effect of pCMB due to its inactivation of inhibitor and release of inhibitor-bound pH 7.8 RNase activity. The difference between the specific activity curves of RNase assayed at pH 7.8 in the absence or presence of pCMB does not represent the absolute amount of inhibitor-bound, latent pH 7.8 RNase activity. Whereas from day 22 onward, the total pH 7.8 RNase specific activity remains constant, the amount of inhibitor-bound pH 7.8 RNase activity per mg protein apparently increases progressively from 0% at day 20 to 6% at day 22, to 30% at 3 months, of the total pH 7.8 RNase activity. This progressive increase in pCMB activation of RNase activity assayed at pH 7.8 would appear to indicate an increase in the concentration of RNase inhibitor-pH 7.8 RNase complexes and such an interpretation is consistent with the observed concurrent decline in free pH 7.8 RNase specific activity. However, no similar concurrent decline in specific activity of free RNase inhibitor is observed. Hence, the amount of free RNase inhibitor must increase at such a rate as to replenish that utilized in the formation of complexes with free pH 7.8 RNase in order to maintain a constant amount of free RNase inhibitor throughout adulthood. However, the favored interpretation of the progressive increase in pCMB activation of RNase activity assayed at pH 7.8 is that it is

due to a further increase in the inhibitor-bound pH 7.8 RNase/pH 9.5 RNase ratio which is due not to an increase in the amount of inhibitor-bound pH 7.8 RNase but, rather, an age-dependent decline in pH 9.5 RNase activity and hence a concurrent decline in the contribution of the inhibitory effect of pCMB to the total RNase activity assayed at pH 7.8. It follows from this interpretation that the amount of inhibitor-bound pH 7.8 RNase remains constant throughout adulthood, whereas the amount of free pH 7.8 RNase activity and pH 9.5 RNase activity concurrently decline after 22 days.

In summary, on a per gram wet weight basis all three RNase activities attain their maximal values at age 22 days and subsequently decline. This decline is gradual and continuous throughout adulthood in the case of pH 7.8 and pH 9.5 RNase activities. Acid RNase activity, however, exhibits a rapid decline in activity between age 22 and 32 days and, subsequently, remains comparatively constant throughout adulthood.

The specific activity of free pH 7.8 RNase inhibitor in the whole brain of newborn rats is about 50% that of adults. There is a rapid increase in free RNase inhibitor activity between age 10 and 18 days and the level of activity attained by 18 days remains constant throughout adulthood. Comparison of the developmental profiles of free RNase

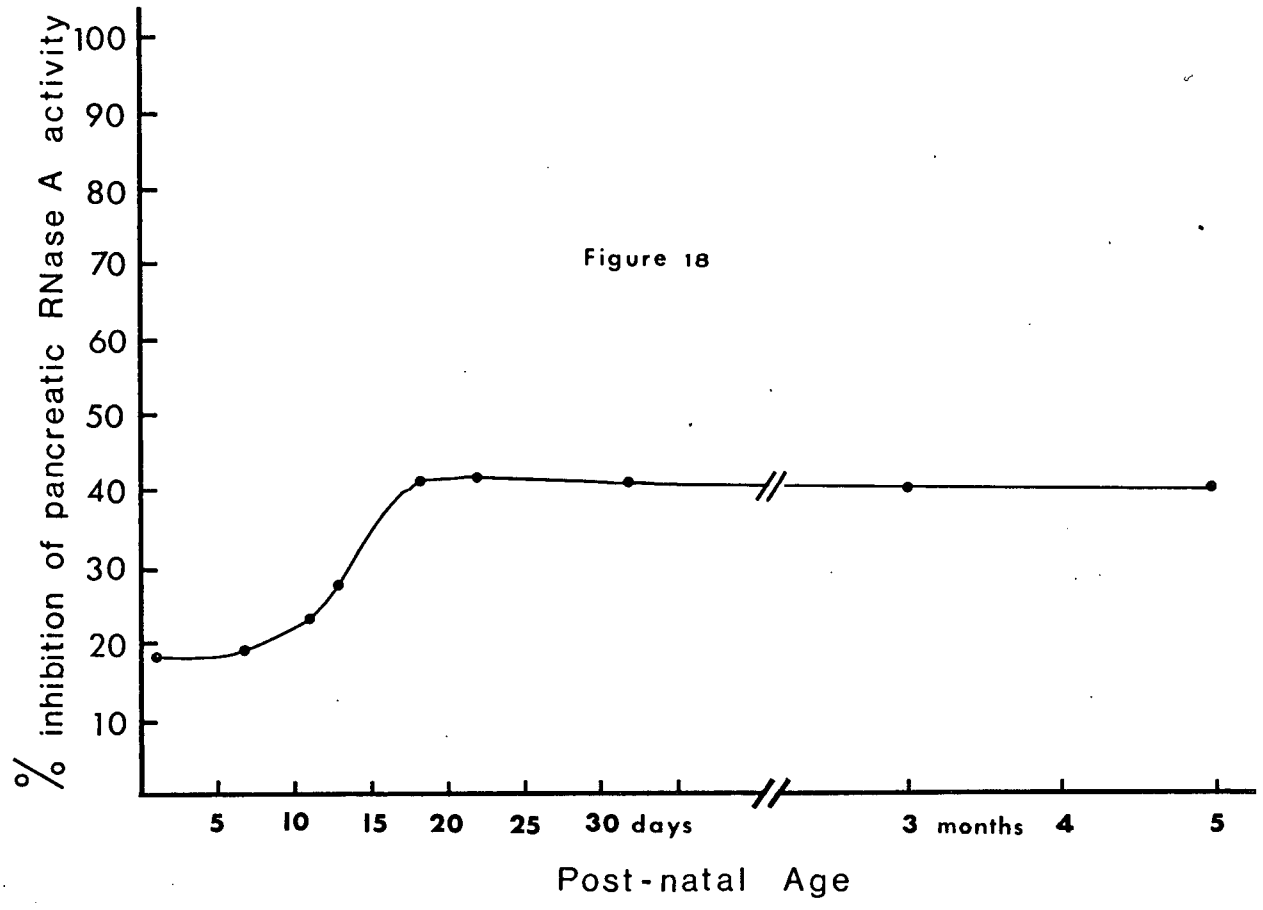

FIGURE 18. Postnatal developmental changes in free RNase inhibitor activity.

Homogenates of 0.1% Triton X100 were diluted with ice-cold .02 M Tris-HCl, pH 7.8 buffer to a protein concentration of 2.0 mg/ml, and 0.2 ml aliquots containing 0.2 mg protein were assayed for inhibition of the activity of 0.5 ng. pancreatic RNase A in the presence of 1.0 mM EDTA. Incubation was for 60 minutes.

inhibitor activity and free pH 7.8 RNase activity indicates a rapid increase in the free RNase inhibitor activity/free pH 7.8 RNase activity ratio between day 10 and day 18. There is a slight decrease in this ratio between day 18 and day 22 since free RNase inhibitor activity has attained its adult level by 18 days whereas free pH 7.8 RNase activity continues to increase up to 22 days. After age 22 days this ratio again increases and continues to increase throughout adulthood due to the gradual decline in free pH 7.8 RNase activity.

Suzuki and Takahashi[285] have studied the developmental change in free RNase inhibitor activity in the cytosol fraction of rat cerebral cortex. These investigators found that this component of the RNase enzyme system exhibits a sharp peak in specific activity between the 5th and 10th day after birth, falls to near neonatal levels by day 13 and remains relatively constant thereafter. However, in the present study using whole cell homogenates of whole brain, free RNase inhibitor specific activity exhibited low levels up to age 10 days after which it increased rapidly and plateaued at adult levels by 18 days. Two of the most obvious explanations for this apparent discrepancy are that (1) there occurs an age-dependent intracellular redistribution of free RNase inhibitor such that the developmental profile of this activity in the

cytosol is not characteristic of the developmental profile of free RNase inhibitor specific activity in the whole cell homogenate, and/or (2) there are region-specific differences in the developmental pattern of free RNase inhibitor activity. The composite results obtained using whole rat brain may not be representative of the developmental changes in free RNase inhibitor activity in different brain regions. If this second explanation is the correct one, then there is a striking and, no doubt, functionally significant difference between the cerebral cortex and the rest of the brain in the developmental profile of free RNase inhibitor specific activity. A study of developmental changes in this component of the RNase enzyme system in different regions of the brain such as the cerebral cortex, cerebellum and brainstem may yield further insight into the role of RNase inhibitor in RNA metabolism and into the functional importance of its presence in such high concentration in brain. Takahashi and Suzuki[285] have reported significant differences in the specific activity of free RNase inhibitor in different regions of the adult rabbit brain.

It must be emphasized that a developmental increase in enzymatic activity measured <u>in vitro</u> does not prove increased enzyme function <u>in vivo</u> where enzyme activity may be regulated by substrate accessibility and other control factors. Developmental increases in enzyme activity do not necessarily

indicate a specific increase in enzyme amount since increased activity could also result from a non-specific increase in the rate of protein synthesis, activation of precursors (activation of bound or latent forms of the enzyme) or to a decrease in the rate of degradation of the enzyme. Whether the observed changes in enzyme activity during development signify an actual increase in enzyme protein due to an enhanced rate of de novo enzyme synthesis, or to developmental changes in factors regulating the activity of a constant amount of enzyme, remains to be shown. Also, it must be stressed that levels of RNase activity expressed in 0.1% Triton X100 homogenates are an index of the total RNase capacity of the tissue. Functional levels of RNase activity in the cell may be more closely approximated by the level of activity expressed in isotonic sucrose homogenates.

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IV. DISCUSSION

The presence of multiple enzymes responsible for the degradation of RNA in brain is consistent with the heterogeneity of this enzyme system in other mammalian tissues and in procaryotes where in the case of wild-type E. coli at least seven distinct RNases have been reported. The true complexity of this enzyme system in mammalian tissues has yet to be elucidated. It is likely that the RNases characterized in the present study, as well as those characterized by other investigators in other mammalian tissues, are the most readily detectable components of an enzyme system consisting of many other additional enzymes of various specificities capable of cleaving phosphodiester bonds in RNA. All of the enzymes identified in the present investigation probably function in the exo- and/or endonucleolytic cleavage of RNA. Highly specific endoribonucleases which are thought to participate in the maturational processing of precursor transcription products into functionally mature RNA molecules would not contribute to the findings reported here since such activities would not be expected to yield sufficient acid-soluble products to be detectable by the assay employed in the present study.

4.0 Regulation of the *In Vivo* Function of RNases in brain

The fact that a large fraction of the detergent-extractable RNase activity is not expressed in assays of isotonic sucrose homogenates indicates that considerable amounts of the maximal levels of all three RNases are present in the cells of the brain in a latent, non-functional state. *In situ*, latent RNase may be present bound to and/or compartmentalized within subcellular organelles thus rendering the enzymes inaccessible to and inactive against substrate. This is supported by intracellular distribution studies which have shown that while the crude mitochondrial fraction contains only 16% of the total cellular RNA,[311] 52% of the recovered total cellular RNase activity is localized in this fraction. Moreover, the microsomal fraction which contains 37% of the total cellular RNA[311] contains comparatively little of the total expressed cellular RNase activity. These observations imply that the functional levels of activity of large amounts of the cellular RNA-depolymerizing enzymes is controlled by restrictions upon enzyme-substrate interaction due to the segregation of enzyme and substrate molecules into different intracellular membrane compartments. It also follows that modulations of the steady state rate of RNA catabolism in the intact cell may be affected by factors altering organelle membrane permeability or shifting the ratio of bound to free enzyme.

Several workers[295,305] have reported that acid RNase in rat liver is associated with lysosomes and exhibits the characteristic structure-linked latency of other lysosomal acid hydrolases. A significant fraction of the total cellular pH 7.8 RNase and pH 9.5 RNase activities have also been found in lysosomes in liver. In view of the similarities of the three RNase activities found in the present study to those of rat liver, it seems likely that a large portion of the total activity which is recovered in the crude mitochondrial fraction of rat brain may also be associated with the lysosomal components of this fraction.

The compartmentalization of acid hydrolases within lysosomes may serve to regulate their activity and prevent the indiscriminate action of these potentially destructive enzymes. In view of the protective function of the lysosomal membrane any injury to the membrane resulting in increased permeability would be expected to enhance the rate of catabolism of cytoplasmic constituents. That segregation of acid RNase within lysosomes is an important mode of regulating the activity of this enzyme in the intact cell is in agreement with the high level of acid RNase in the cell, the absence of any known specific inhibitors of acid RNase, and the inactivity toward this enzyme of pH 7.8 RNase inhibitor. Hence, a discussion of the regulation of the <u>in vivo</u> activity of acid RNase must take into consideration the mode of action

of lysosomal enzymes and the regulation of lysosomal function.

Although the presence of acid hydrolases within lysosomes is well established, their site of function is still in dispute. Two hypotheses have been formulated as to how lysosomes perform their digestive functions in the intact cell. According to one, material is encapsulated by membrane to form endocytotic vacuoles which then fuse with lysosomal membranes and empty their contents into the lysosome where they are degraded and the degradation products recycled to the cytosol. According to this model lysosomal contents normally remain continuously delimited by an uninterrupted membrane which shields the rest of the cytoplasm against attack by lysosomal enzymes. This hypothesis is supported by observations that (1) phagocytized and pinocytized material is found in lysosomes; (2) primary lysosomes fuse readily with endocytotic vacuoles[312,313] as well as with other lysosomes (but not with mitochondria or nuclei),[314,315] and (3) an intralysosomal site of action for lysosomal enzymes, most of which have acid pH optima, is compatible with normal intralysosomal pH which has been reported to be 6.34 for rat liver lysosomes.[316]

According to the second hypothesis, the enzymes compartmentalized within lysosomes are normally inactive and participate in the catabolism of cell constituents only upon

release into the soluble cell sap.[317] Thus, only lysosomal enzymes which occur free in the cytosol represent metabolically available enzyme. Support for this hypothesis comes from several lines of investigation. It has been observed[318-321] that increased fragility of lysosomes is correlated with enhanced levels of various acid hydrolases in the cytosol. This implies release or leakage of lysosomal acid hydrolases into the cytosol and this process appears to be more pronounced under conditions of cell stress than during normal cell functioning. Some forms of stress that have resulted in an increase in acid RNase activity of the cytosol in liver cells include hypophysectomy,[322] adrenalectomy,[322] and carcinogenesis.[194] Furthermore, the release and activation of acid hydrolases into the cytosol has been reported to precede and contribute to cell degeneration during viral infection.[329] An increase in the ratio of free to bound lysosomal enzyme activity during tissue degeneration has also been reported.[256-258, 323-325]

Finally, several reports[326-328] indicate that the activity of lysosomal enzymes increase with cell age. Lysosomal membrane lysis and/or acid hydrolase induction under conditions of extreme cellular (metabolic and functional) stress may thus be important mechanisms in the process of cell aging and autolysis. The role of lysosomal

RNases and other acid hydrolases in tumor regression and cell degeneration may, however, be secondary to a general enhancement in the rate of cell or tissue catabolism.

Although acid hydrolases would be expected to have much reduced activity at the normal physiological pH of the cytosol, some conditions of cellular stress under which fragmentation of lysosomes may occur are accompanied by a concomitant acid shift in the normal pH of the cytosol (e.g., hypoxia, eschemia,[330] acidosis, neoplasm[358]) thus resulting in more favorable conditions for the degradation of cytoplasmic constituents by lysosomal enzymes.

A method that has been used to gain information regarding the function and site of action of lysosomal enzymes has been the physiological perturbation of intact animals. Thus, Pontremoli et al.[331] have reported the occurrence of increased numbers of lysosomes, changes in their morphology including membrane breaks, as well as release into the cytosol of several lysosomal enzymes in the hepatocytes of starved or cold-stressed rabbits. If such a phenomenon represented a general mechanism of metabolic response to cellular stress, it would be expected that cytosol levels of acid RNase would also be increased in starvation or cold-stressed cells. However, such results have not been reported. De Lamirande and Allard[202] have, in fact, reported that fasting slightly

lowers the levels of both acid RNase and pH 7.8 RNase activity without altering the intracellular distribution of these enzymes in rat liver. Deckers-Passau et al.[332] have reported differential reactions of several lysosomal enzymes to changes in physiological state of the animal.

Dynamic changes in membrane permeability may be of critical importance in the regulation of in vivo RNase activity since such changes will determine the accessibility of these enzymes to their substrates. The key to understanding the role of lysosomal enzyme function in cellular metabolism may thus lie in identifying those factors responsible for maintaining the structural and functional integrity of lysosomal membranes or affecting lysosomal membrane permeability thereby facilitating or inhibiting the release of lysosomal enzymes into the cytosol. In this regard, Demin and Nechaeva[333] have shown that adrenalin at 3.3×10^{-3}M causes a 27% activation of acid RNase activity in cerebral cortex crude mitochondrial fraction incubated in isotonic sucrose, whereas acetylcholine at 6×10^{-6} M inhibits the normal leakage of latent acid RNase from this subcellular fraction. In leucocyte lysosomes, however, catecholamines have been shown[334-339] to inhibit lysosomal enzyme release and this effect has been thought to be mediated through enhanced levels of intracellular cAMP. Cyclic AMP has also been reported to increase the permeability of lysosomal membranes

with respect to acid phosphatase and ß-glucuronidase in rat liver[340] and to acid RNase during degeneration of hormone-dependent mammary tumors.[256]

Some of the discrepancies between these findings may be explained in terms of tissue-specific differences in the response of lysosomes to these reagents. Also, since lysosomes have been shown[201,341,342] to be heterogeneous in their size and enzyme content, it is possible that variable activation or release of lysosomal enzymes may be due to selective membrane permeability changes in specific classes of lysosomes or to differences in permeability of the lysosomal membrane to specific acid hydrolases.

The implication of cAMP in the regulation of lysosomal membrane permeability combined with reports of neurotransmitter-stimulated adenyl cyclase in brain suggests a plausible mechanism by which the activation of post synaptic cell surface neurotransmitter receptors may modulate neuronal RNA metabolism. Oscillatory changes in neuronal RNA content accompanying electrophysiological activity[154] may thus be attributable to the intracellular release of a receptor-transduced metabolic demand signal which modulates the rate of RNA degradation by regulating RNase accessibility to substrate.

There have been consistent findings of a positive correlation between elevated RNase activity or decreased RNase inhibitor to pH 7.8 RNase activity ratio and either reduced rates of protein synthesis or a general increase in catabolic activity. This is in agreement with the view that functional levels of these enzymes may limit the availability and frequency of translation of mRNA molecules. Furthermore, the finding that reduced levels of free RNase inhibitor or enhanced levels of free pH 7.8 RNase activity is correlated with enhanced polysome breakdown, decreased capacity of ribosomes for accepting or translating mRNA, decrease in functional mRNA, and general impairment in the functioning of the translation apparatus suggests that RNase inhibitor may play a significant role in regulating protein synthesis by preserving the structural and functional integrity of polysomes.

Several workers have investigated the disaggregation of isolated polysomes. Eker and Pihl[343] have reported that the disaggregation of polysomes <u>in vitro</u> and the time-dependent activation of a latent RNase activity associated with polysome preparations is prevented by 1.0 mM dithiothreitol or 1.0 mM glutathione. The effect of these compounds was not due to a direct inhibiting effect on the enzyme since these compounds do not significantly inhibit the activity of

partially purified RNase preparations which exhibit neither stimulation by pCMB nor inhibition of bovine pancreatic RNase A. It has also been reported that rat liver cytosol stabilizes polysomes,[344-346] m RNA,[345,347] and HnRNA[348] in vitro. RNase inhibitor has been demonstrated by several workers[222,345,357] to stabilize polysomes and prevent their disaggregation. The presence of excess free RNase inhibitor in the cytosol has thus been assigned the physiological role of stabilizing the translation apparatus by preventing the degradation of polysomal RNA.

The release and activation of inhibitor-bound pH 7.8 RNase could be effected by any endogenous factor capable of destabilizing the inhibitor-RNase interaction and dissociating the inhibitor-RNase complex. Factors such as reduced sulfhydryl compounds capable of modulating the interaction between free RNase inhibitor and free pH 7.8 RNase could shift the dynamic equilibrium between RNase-inhibitor complexes, free pH 7.8 RNase, and free RNase inhibitor, thereby regulating the rate of RNA degradation. The equilibrium between these molecular species could also be controlled by constraints to interaction between any two components. Thus, the intracellular distribution data indicate that the compartmentalization of free RNase inhibitor may be of importance in determining the differential

rates of RNA degradation in the cell nucleus and cytoplasm. The absence of free RNase inhibitor activity in the nucleus indicates that the nucleo-cytoplasmic envelope functions as a permeability barrier which prevents the large excess of free RNase inhibitor in the cytosol from equilibrating with the nucleoplasm.

It must be emphasized that the levels of RNase activity measured under optimized conditions *in vitro* cannot be taken as a reliable index of enzyme function in the intact cell. The activity of these enzymes under normal physiological conditions is not known. The contributions of each of the three RNases identified in this study to cellular RNA degradation under normal physiological conditions is also not apparent. Information as to the preferential substrate specificities of each of the three enzymes with respect to different classes (e.g., rRNA, tRNA, HnRNA) of brain RNA may provide a better understanding of their function *in vivo*.

In view of the facts that (1) there is no detectable free pH 7.8 RNase activity in the cytosol; (2) pH 6.7 RNase exhibits negligible activity at pH 7.4 and is markedly inhibited by physiological tonicity above pH 6, and (3) pH 9.5 RNase exhibits only 68% of its optimal activity when assayed at pH 7.8 and is also markedly inhibited by physiological ionic strength, it would seem that intracellular RNA would

be protected from the action of these enzymes under normal physiological conditions. The measured levels of RNase activities hence probably represent total cellular capacity for RNA degradation and do not reflect functional levels of RNase activities participating in the breakdown of RNA molecules under normal physiological conditions *in vivo*. It is, nevertheless, clear that certain perturbations in the steady-state physiological conditions could result in an activation of the latent RNA-degrading capacity of brain cells and hence in an enhanced rate of RNA degradation.

4.1 Correlation of Developmental Changes in the Content, Synthesis and Degradation of RNA in brain

Despite previously mentioned reservations, it seems worthwhile to make some tentative inferrences as to the possible functional significance of the developmental changes in RNase activities observed under the conditions of the present study. How are these changes correlated with developmental changes in RNA synthesis and cellular RNA content?

Figure 19 is a composite developmental profile of the total RNA degradative capacity of whole brain constructed by summing the experimentally measured activities of each individual RNase (pH 6.7 RNase, pH 9.5 RNase, and free pH 7.8 RNase) at a given age.

The increase in total cellular RNA hydrolytic capacity per gram wet weight of whole brain between 10 and 20 days

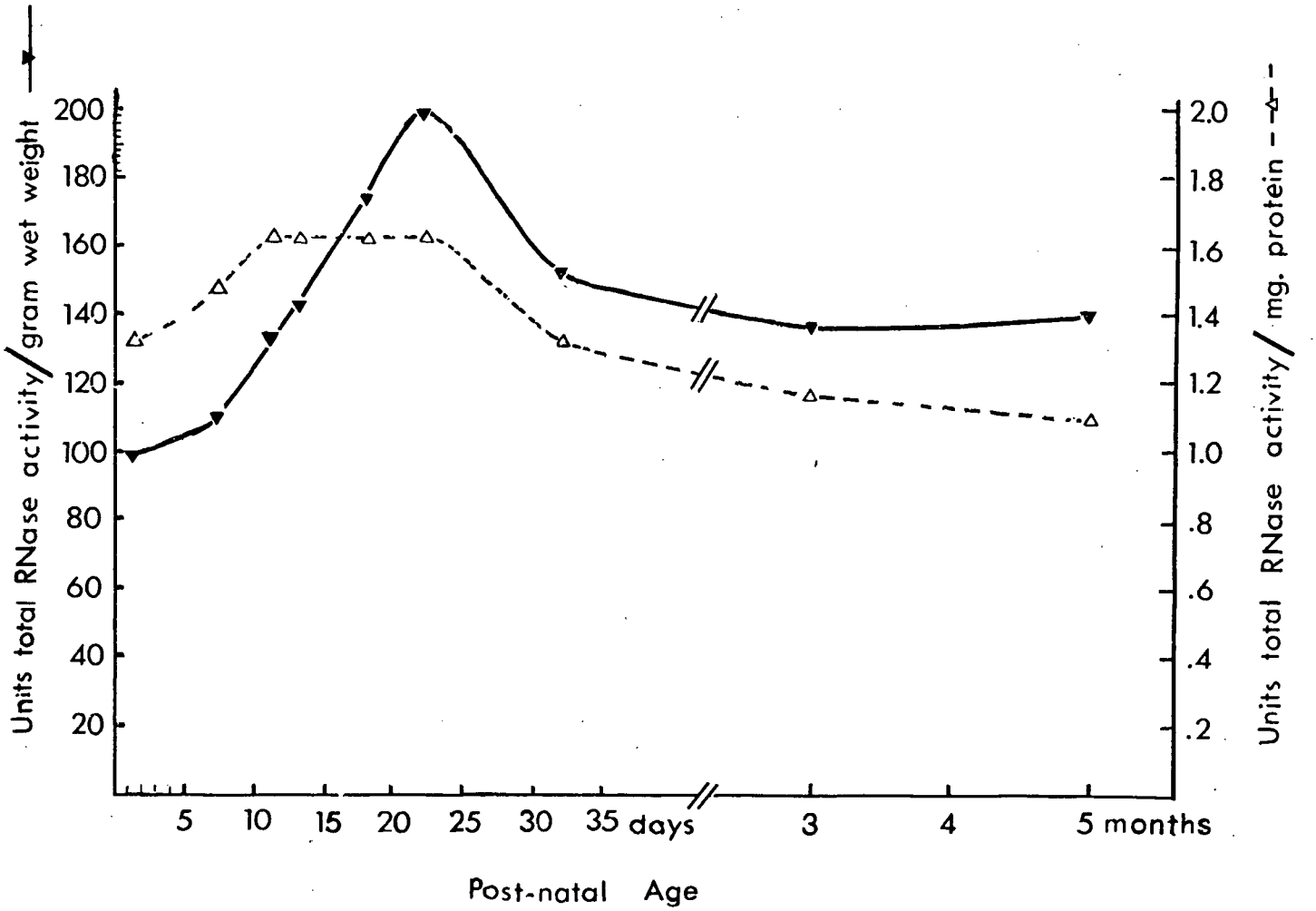

FIGURE 19. Total RNA degradative capacity of whole brain as a function of the postnatal age of rat.

This is a theoretical curve constructed from data shown in Figures 15, 16 and 17 by summing the activity of each individual RNase assayed at pH 6.7, 9.5 and 7.8 (free pH 7.8 RNase activity) at each age.

would be expected to correspond to an increase in the *in vivo* rate of RNA degradation, unless it is accompanied by a proportional increase in regulatory mechanisms restricting the activity of RNases. This increase in brain RNA degradative capacity directly parallels the concurrent increase in organ and cellular RNA content for it has been shown that the total RNA content of both the cerebral cortex and whole brain continues to increase up to age 20 days at which time this accumulation ceases. Hence, the rate of total RNA synthesis exceeds the rate of total RNA degradation by a decreasing increment which approaches zero by age 20 days when a steady state balance between the rates of RNA synthesis and degradation is attained and a constant organ content of RNA is maintained.

Possibly the most reliable data on the rate of total RNA synthesis is that obtained with tissue slices. Unfortunately, developmental data for the rate of total RNA synthesis in whole brain slices is not available. However, developmental studies of the rate of RNA synthesis in rat cerebral cortex slices[46,349] indicate that the rate of synthesis declines (undergoing a 3 to 4-fold decrease) after age 10 days and attains adult steady state levels by about 20 days.

Since the rate of total RNA synthesis declines during the time when organ RNA content is accumulating and the total RNA degradative capacity (per gram wet weight whole brain) is increasing, it follows that any concomitant increase in the rate of RNA degradation is limited by the fact that the total rate of RNA degradation does not exceed the declining rate of RNA synthesis.

Since the steady state adult rate of RNA synthesis and the final organ and cellular RNA content is attained by age 20 days and subsequently sustained, it also follows that the rate of total RNA degradation and hence the functional level of RNase activity must also reach steady state conditions by this age. However, after attaining peak levels at 20 days, the RNA-degradative capacity of whole brain subsequently declines up to age 30 days. Since between 20 and 30 days there is no net accumulation in organ RNA, and since the rate of RNA synthesis remains constant, it must be tentatively concluded that the decline in maximal RNA-degradative capacity or maximal RNase activity is not accompanied by a parallel decline in the functional contribution of these enzymes to the rate of total RNA degradation *in vivo*. Thus, although the total RNA-degradative capacity declines between age 20 and 30 days, compatibility with the other events observed during this time requires that the amount of this capacity which is functional *in vivo* must remain constant after 20

days. Control factors must regulate and maintain a constant level of RNase activity after age 20 days despite the decline in total RNase capacity. Hence, an increasingly greater fraction of the total RNA-degradative capacity or maximal RNase activity is functional *in vivo*. For the functional or expressed level of RNase activity to remain constant while the maximal RNase activity measured *in vitro* decreases, there must occur either a preferential dropout in the amount of non-functional, latent, RNase activity and/or a proportional decline of both functional and non-functional RNase (activity) concomitant with a progressive relaxation of control factors normally restricting the expression of RNase activity. This might be thought of as increasing the *in vivo* specific activity of the enzymes.

Elucidation of the precise relationship of the developmental changes in the measured levels of RNase activity to the changes in the physiologically functional levels of activity of these enzymes will require a developmental study of the rate of turnover of total RNA in whole brain. Such data would provide a more reliable index of the *in vivo* rate of total RNA degradation. The difference curve between the developmental profiles for total RNA turnover and for total RNA-degradative capacity would then represent developmental changes in control factors regulating the expression of maximal RNase activity.

4.2 Regional Differences in the Metabolism of RNA and in the Functional Roles of RNases in brain

The developmental pattern of RNA depolymerase activities can be correlated with the occurrence of both quantitative and qualitative changes in the pattern of RNA synthesis during brain development. On both a per gram wet weight brain and per milligram protein basis neonatal rat brain contains a higher amount of pH 6.7 RNase activity than adult rat brain. Older rats have higher levels of both alkaline RNase activities. The higher titer of acid RNase during the cell proliferation stage of brain development suggests a possibly specific role for this enzyme in the catabolism of RNA during cell division. Presumably, RNA metabolism during cell differentiation entails a rapid degradation of RNA and the synthesis of new species of RNA molecules. The rise in alkaline RNase activities between day 7 and day 22 may be associated with the replacement of the mRNA molecules coding for mitosis and cell differentiation by RNA molecules required for the maintenance functions of non-dividing brain cells. Thus, these enzymes may participate in the turnover of the RNA content of fully-differentiated cells.

This line of reasoning gives rise to the expectation that significant differences in the developmental profiles

of these RNase activities may occur in different brain regions. Unlike the cerebral cortex in which cell division is almost complete at birth, the cerebellum in the rat exhibits rapid cell proliferation up to 16 to 20 days after birth.[47,308,309] Hence, since acid RNase activity is highest during the cell proliferation stage of brain development, the decline in specific activity of acid RNase may occur at a later postnatal age in the cerebellum than in the cerebral cortex. Also, the rise in alkaline RNase activities might be expected to begin at a later age in the cerebellum and/or to be more prolonged than in the cerebral cortex.

Such regional differences in the levels of the various components of the RNase enzyme system during maturation may account for the finding that developmental changes in RNA metabolism in the cerebral cortex are not representative of whole brain. The available information[24,95,350] indicates that the total RNA content of the cerebral cortex, unlike the cerebellum and whole brain, actually declines after attaining peak values at 18 to 20 days of age, and this decline appears to be due to a preferential depletion of rRNA.[24] Since it has been shown[46,349] that the rate of RNA synthesis in rat cerebral cortex slices declines up to 20 days and subsequently remains constant, the decline in RNA content of this brain region after age 20 days must be due to an increase in the rate of RNA degradation.

This conclusion is in agreement with the difference in the developmental profiles of free RNase inhibitor specific activity found here for whole brain and that reported by Takahashi and Suzuki[285] for cerebral cortex. The lower free RNase inhibitor specific activity in the cerebral cortex of adult rats as compared to 10 day old animals is consistent with an enhancement in the rate of RNA degradation in this brain region.

In the adult rat the cerebral cortex contains more RNA per average cell[23] and has a 2-6 fold greater rate of RNA synthesis[46] than the cerebellum. It follows that at this age RNA turnover and the total RNA degradative activity will be proportionally greater in the cerebral cortex as compared to the cerebellum. The difference in the rate of RNA degradation between these two areas will be greater than the difference in the rate of RNA synthesis such as to allow a net depletion of RNA content in the cerebral cortex after age 20 days.

Direct testing of these conclusions will require a comparative study of the developmental changes in RNA turnover in these brain regions. Dawson[71] studied the rate of rRNA turnover in 35 day old rat brain after an intracerebral injection of labelled uridine and found that the label incorporated into rRNA decayed with a half-life of 6 days. Studies

using other routes of precursor administration have shown that in adult rat, whole brain rRNA turns over with a half life of 12 days.[78,351] It seems likely that intracerebral injection of precursor in Dawson's study may have resulted in the preferential labelling of rRNA in the cerebral cortex, and hence, the higher turnover rate of rRNA reported by Dawson may reflect a higher rate of rRNA synthesis and degradation in the cerebral cortex as compared to other regions of the adult rat brain. The fact that for whole brain the rate of rRNA turnover in the newborn rat is twice that of the adult[70] is consistent with the developmental pattern of free RNase inhibitor activity observed for whole brain in the present study. If free pH 7.8 RNase participates in the degradation of rRNA, the higher level of free RNase inhibitor in the brain of the adult would limit the activity of this enzyme thus affording a longer half-life of RNA.

Although it was desirable to obtain an overall picture of the RNase activities of the whole brain, it must be stressed that the data thus obtained may mask significant region-specific differences. In view of the organizational complexity of the brain and the differential rates of development of different brain regions, a regional distribution study will be required to test some of the speculations which have emerged in the foregoing discussion. Moreover, the data

obtained in the present study of rat whole brain represents composite results for a complex mixture of cells (primarily neurons and glia) each of which may have quite different degradation capacities with respect to RNA.[59] For example, Watson[90] has reported that RNA turnover proceeds more rapidly in neurons than in glia and neuronal RNA turnover is markedly subject to variation depending on the environmental status of the animal. It is hence desirable that measurements of the functional levels of the various components of the RNase system be extended to localized brain regions and homogeneous cell populations of discrete cell types.

4.3 Tissue-specific Differences in RNA Turnover and RNase Activities in the adult animal

In the adult rat rRNA of whole brain turns over at half the rate of liver rRNA. This is consistent with the observation that when expressed on a per gram wet weight basis the liver content of RNase activity is 10-fold higher than brain and its inhibitor-bound pH 7.8 RNase is 2-fold lower than brain.

Although the level of free RNase inhibitor activity in rat liver was not measured in the present study, Roth[187] has reported that in adult rats the specific activity of free

RNase inhibitor of the cytosol fraction is 15% higher in whole brain than in liver. Roth also found that the specific activity of free RNase inhibitor in the cytosol of liver was 2-fold greater than that of kidney and in accordance with this the specific activity ratio of free pH 7.8 RNase in the cytosol of kidney to liver was 40:1. As would be expected, the specific activity ratio of total pH 7.8 RNase (assayed in the presence of pCMB) was considerably lower (4:1) than this. De Lamirande and Allard[202] in a comparative study of free pH 7.8 RNase and acid RNase activities in different tissues of the adult rat found lowest levels of both activities in the cerebral cortex (3-fold lower than in liver). Greenstein et al.[352] and Roth and Milstein[353] have also reported that the acid RNase activity of whole brain is amongst the lowest of all rat tissues studied. Ellem and Colter[215] studied the levels of acid RNase and free pH 7.8 RNase activities in various mouse tissues. The activity of acid RNase per gram wet weight tissue was 3-fold lower for brain than for liver and the activity of free pH 7.8 RNase per gram wet weight tissue was 4-fold lower for brain as compared to liver. The level of both enzyme activities on a per gram wet weight tissue basis was higher in kidney than liver and lower in muscle than brain.

The significance of these tissue-specific differences in RNase activities is not known. However, the lower level of total RNase activity in whole brain, as compared to liver of adult rats, may be relevant to the fact that in the adult rat rRNA of whole brain turns over at half the rate of liver rRNA. A better understanding of the functional significance of the higher level of inhibitor-bound pH 7.8 RNase and possibly also of free RNase inhibitor activity in brain, as compared to liver, must await further information.

4.4 Concluding Remarks

The present study has elucidated some of the gross features of the enzyme system which participates in the degradation of RNA in brain. The RNase enzyme system in brain is similar in its broad and general outline to that studied in other mammalian tissues and may differ from other organs only in the sophistication and versatility of the regulatory mechanisms by which its activity is controlled and coordinated with other enzyme systems. The initial intent of this study was to explore the RNase system in brain for any distinguishing features which might be specifically related to the specialized functions of this tissue. However, a preliminary characterization of the properties of this enzyme system in whole brain was required due to the lack of information as to its RNase content and composition. A

deeper level of analysis will be required to evaluate the physiological function of these enzymes and their respective roles in the integrated metabolism of RNA in brain.

The effect of a number of variables influencing the determination of the component activities of the RNase system has been clarified. This information thus provides the necessary groundwork for the investigation of variations in the functional levels of the various components of this enzyme system following alterations in the milieu of the animal or alterations in specific brain functions. RNase activities and RNase inhibitor levels can now be measured in brain following various physiological stresses such as learning experience, sensory deprivation, electroschock, malnutrition, as well as during cell degeneration following axonal section or electrolytic lesions. Such measurements of the functional levels and subcellular distribution of the various components of the RNase system under conditions known to be accompanied by changes in cellular RNA content may provide a clearer understanding of the contribution of each of the component activities to the specific changes in RNA metabolism and cellular RNA content which have been reported to occur in brain. This would result in a clear definition of the complex relationship between the activity of each of

the identified components of this enzyme system to neuronal functional states.

Further information is required to determine (1) the significance of the high level of RNase inhibitor in brain and how it is geared to specific tissue function; (2) how regulatory mechanisms controlling RNA catabolism are coupled to the electrophysiological activity of neurons, and (3) how these mechanisms respond to changes in the neuron's functional demand for RNA.

To achieve a clear picture of integrated RNA function and metabolism in the intact cell, changes in the rate of RNA degradation must also be correlated with changes in the rate of de novo RNA synthesis, conversion of precursor transcription products into functionally mature RNA, and nucleocytoplasmic transport. The detection of changes in these parameters in response to enhanced electrophysiological activity may be critical to the identification of those molecular events which underlie the modification of a neuron's functional relationship with other neurons.

The present investigation provides a descriptive picture of the baseline RNase composition of rat whole brain under states of normal brain function. It is hoped that the information reported herein will provide guidelines

for prospective investigations into the more basic questions of the nature of the molecular mechanisms and control points through which RNA metabolism is regulated and the underlying molecular processes by which changes in cellular RNA metabolism result in alterations in integrated brain cell functions.

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