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THE SYNTHESIS OF AMYLASE IN PAROTID GLANDS OF YOUNG AND OLD RATS

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SUMMARY

The age-related changes in the rate of synthesis of total and secretory proteins were examined in parotid glands of young (2 months) and old (24 months) rats. The differences in the rate of incorporation of radioactive leucine into acid-insoluble proteins of the gland indicate that the rate of protein synthesis declines with age in this gland. To determine whether the rate of synthesis of secretory proteins changes with age in this gland, the rates of incorporation of [^{3}H]leucine into amylase, a major secretory protein of the gland, were compared by radioactivity determinations. For this comparison, amylase was precipitated with glycogen after incubating the gland slices in the presence of the labeled amino acid. The study shows that rate of synthesis of amylase declines significantly with age in this gland. The possible relationship between the decline in protein synthesis and the reduced level of secretory activity of the gland due to aging is discussed.

Key words: Secretory protein synthesis; Aging

INTRODUCTION

Previous studies have shown that the rate of cellular protein synthesis declines with age in many different organs and tissues (see ref. 1 for review). This decline in protein synthesis may be an underlying cause for the loss of organ or tissue

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functions which eventually leads to the death of the organism [2]. In the case of the salivary gland, reductions seem to occur in the amylase content of the saliva as well as in the rate of flow and secretion of saliva with increasing age [3–5]. The reduced content of amylase in the saliva suggests that decremental changes occur with age which affect the mechanisms of either the synthesis or release of secretory proteins in salivary gland cells.

We have shown previously that, in rat parotid glands, the rate of synthesis of proteins declines with age in the acinar cells [6–8], but not the ability of these cells to release secretory proteins [9]. Our previous analyses of secretory proteins in the postmicrosomal supernatant of the gland by electrophoretic separations have indicated that this decline in protein synthesis involves the reduction in the synthesis of secretory proteins [10,11]. However, the rate of synthesis of specific secretory proteins has not been compared in young and old rats. Furthermore, the rats used in our previous studies were not checked for the possible infection by sialodacryoadenitis (SDA) viruses which are wide spread among commercial stocks of rats and known to affect the functions of rodent salivary glands [12].

To determine whether the rate of synthesis of secretory protein declines specifically with age, we have used SDA virus negative rats and compared the rate of synthesis of amylase, a major secretory protein of the parotid gland, in 2- (young) and 24- (old) month-old rats. The study indicates that the rate of synthesis of this secretory protein is significantly reduced in the older rats as determined by radioactive amino acid incorporation analyses.

MATERIALS AND METHODS

Animals

Male, Sprague–Dawley rats were used in this study and were obtained from the Charles River Breeding Laboratories in Portage, Michigan. The rats were 2 and 24 months old and weighed about 240 g and 600 g, respectively. The older rats were maintained in an aging colony at the above mentioned plant.

All rats were Caesarean-derived and maintained behind a barrier-containment facility. These rats were checked every 4 weeks by serological means for mycoplasmosis, pathogenic organisms, and parasites. Particularly, these rats were monitored regularly for the SDA virus and the rats used in this showed negative titers for this virus. The rats were fed Purina rat chaw which contained 22% protein and 5% fat (Charles River formula). The drinking water was UV treated and ultrafiltrated. Food was withheld for about 16 h before experiments which usually began about 0900 h.

Incorporation of [^3H]leucine into glandular proteins

The rate of protein synthesis was determined as described in our previous studies [6–8]. The parotid gland slices were incubated in Minimum Essential

Medium (MEM: Grand Island Biological Co., Grand Island, N.Y.) without leucine supplemented with 9 μCi/ml of L-[4,5,^{3}H(N)]leucine (spec. act. 47–55 Ci/mmol; New England Nuclear, Boston, MA). The incubation medium was also supplemented with unlabeled leucine to a total concentration of 0.1 mM. After incubation, the tissue slices were homogenized and precipitated with 0.3 N perchloric acid (PCA). The supernatant was used to determine [^{3}H]leucine available in the PCA-soluble pool and the specific activity of the leucine pool. Radioactivity determinations were made in a Beckman 7800 scintillation spectrometer. The rate of incorporation between the age groups was compared based on the DNA content of the gland. The amount of DNA was determined by the diphenylamine method of Burton [14] after extracting it from the PCA precipitate as described previously [6–8].

Size and specific activity of free leucine pool

Amino acid analyses were done by a Kratos amino acid analyzer system equipped with a AA 511 cat ion exchange column (Interaction Co., Los Altos, CA). The reagent was *o*-phthaldehyde, and the amino acids were derivatized in a post-column reactor, and detected flurometrically with a Kratos FS 950 flurometer. The procedure is modification of the method of Böhlen and Mellet [15]. One-minute fractions were collected in scintillation vials with a Gilson FC-100 fraction collector. Each fraction was mixed with Atomlight (New England Nuclear Products, Boston, MA) and counted for radioactivity on a Beckman LS6800 scintillation counter.

Incorporation of [^{3}H]leucine into amylase

After the incubation of the parotid gland slices as described above, the amylase was precipitated from crude extracts of the gland as a glycogen-enzyme complex as described by Loyter and Schramm [13]. Radioactivity in the glycogen precipitate was determined by scintillation counting in a Beckman 7800 spectrometer. For the determination of the amount of [^{3}H]leucine incorporated into amylase, the amount of radioactivity in the glycogen precipitate was adjusted to account for the differences in the recovery of the enzyme by precipitation from assays to assays.

Characterization of the glycogen precipitate

The water soluble proteins of the glandular homogenate and glycogen precipitate were separated by electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate (SDS). The molecular weight of the amylase precipitated with glycogen was estimated by comparing its electrophoretic mobility to that of the several proteins of known molecular weight [16]. The enzyme activity of amylase after glycogen precipitation was determined as described below and the specific activity of the enzyme was compared in young and old rats.

Assays of amylase activity

The amylase activity was assayed by the procedure used previously in our earlier work [6,7,9]. Briefly, the glandular amylase was extracted by an extensive homogenization in distilled water or phosphate buffered saline (PBS) and the enzyme activity was determined by using the Amylochrome method (Roche Diagnostics, Nutley, N.J.). This method measures the amount of dye released from the Cibachrone Blue F3 GA-amylose complex when the 1–4 bonds of amylose are broken by the action of amylase.

RESULTS

The rate of incorporation of leucine into the PCA-insoluble fraction of the parotid gland is about 30% lower in 24-month-old rats than in 2-month-old rats (Table I), indicating that the rate of total protein synthesis declines with age. The difference in the rate of incorporation at these ages does not appear to be due to the changes in the size of the precursor pool with age. The amino acid analysis of the free leucine pool in parotid glands indicates that there is no difference in the size of this precursor pool at 2 and 24 months (Table II). However, the amount of radioactivity in the leucine pool of the older rats is about two times as much as that in the younger ones (Table II), as is expected from the lower rate of incorporation in the older group.

To compare the rate of synthesis of amylase, the rate of incorporation of [^{3}H]leucine into this secretory enzyme was determined. In the glands of the young and old rats, the incorporation of [^{3}H]leucine into amylase occurs at a constant

TABLE I

THE AMYLASE CONTENT AND THE RATE OF LEUCINE INCORPORATION INTO ACID-INSOLUBLE PROTEIN IN PAROTID GLANDS

The data represent mean ± standard error of means.

Age (months)	*Amylase content [dye Units*/mg DNA (×10^{-6})]*	*Leucine incorporation (nmoles**/mg DNA/h)*
2	40.30 ± 2.80 (4)	140.0 ± 5.1[a] (5)
24	47.46 ± 3.00 (3)	96.0 ± 4.8[a] (8)

*Dye units refer to the number of dye units released from Cibachrone Blue-amylose complexes upon hydrolysis of the bond by amylase. 1050 dye units equal 1 mg maltose produced from starch in 3 min of incubation at 37°C (1 unit of amylase activity). The values regarding the amylase content at 2 and 24 months are not significantly different.

**The amount of leucine was calculated from the amounts of [^{3}H]leucine incorporated into the acid-insoluble fraction following the incubation of the gland slices as described in the Method section.

[a]These two values are significantly different ($P < 0.001$). The number in parentheses refers to the number of rats used for the assay in each case.

TABLE II

THE SIZE AND SPECIFIC ACTIVITY OF FREE LEUCINE POOL IN PAROTID GLANDS OF YOUNG AND OLD RATS

The data represent mean ± standard error of means for 3 separate assays. The intracellular free leucine was fractionated and radioactivity was determined as described in the Materials and Method section.

Age rats	*Number rats*	*nmoles/ mg DNA*	$\frac{cpm}{nmole} \times 10^{-4}$
2	2	13.93 ± 2.83[a]	8.00 ± 0.13[b]
24	2	19.42 ± 0.34[a]	14.30 ± 0.18[b]

[a]Values are not different significantly ($0.2 < P < 0.1$).
[b]Values are different significantly ($P < 0.001$).

rate for 1 h (Fig. 1), as determined by radioactivity counting of the enzyme after precipitating with glycogen. The rate of incorporation of the amino acid is significantly lower (about 40%) in the older rats than in the younger counterparts when compared based on the DNA content of the gland, (Table III).

The amylase precipitated with glycogen for radioactivity determinations forms a single band upon electrophoresis on polyacrylamide gel containing SDS and co-migrates with a prominent band of glandular protein with 56 000 molecular weight (Fig. 2). The glycogen-precipitated amylase, which amounts to about 15%

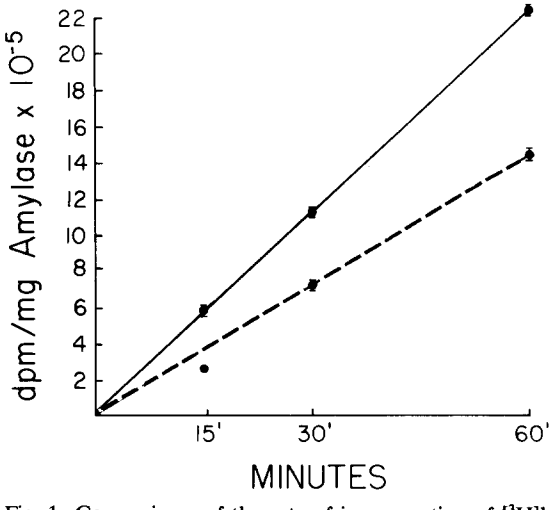

Fig. 1. Comparisons of the rate of incorporation of [^{3}H]leucine into parotid amylase in 2- (solid line) and 24- (dotted line) month-old rats. After incubating the gland slices in the presence of the labeled amino acid for the indicated 3 time periods, amylase was precipitated with glycogen from the gland homogenate for radioactivity determinations by scintillation counting. The incorporation of leucine into amylase occurs at a constant rate for 1 h in both age groups. Each point represents the mean ± standard error of means from 3 separate experiments.

TABLE III

THE INCORPORATION OF [^{3}H]LEUCINE INTO AMYLASE IN PAROTID GLANDS OF YOUNG AND OLD RATS

The data represent mean ± standard error of means. The difference in the incorporation values in these ages is significant ($P < 0.01$). The amount of radioactivity incorporated into amylase was determined after extracting the enzyme as the glycogen precipitate from the homogenate of the gland slices. The gland slices were incubated in the presence of the labeled amino acid prior to glycogen precipitation of amylase.

Age months	*Number rats*	*Number samples*	$\frac{dpm}{mg\ DNA\ h} \times 10^{-6}$
2	6	10	2.020 ± 0.169
24	6	3	1.265 ± 0.092

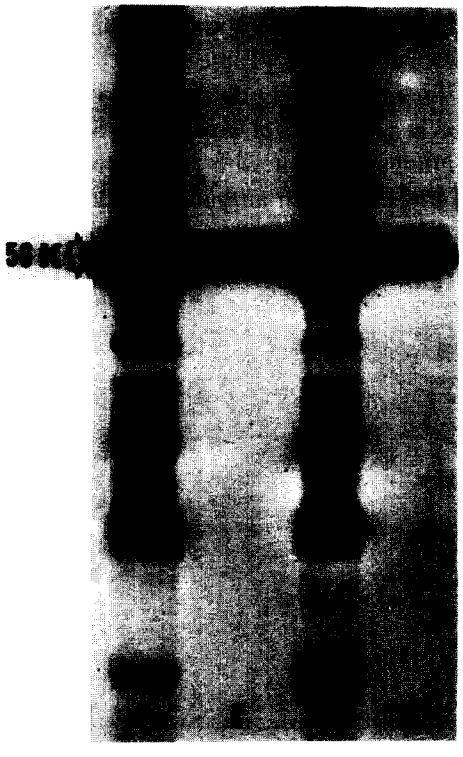

Fig. 2. SDS-polyacrylamide gel electrophoresis of the glandular homogenate and amylase precipitated with glycogen from parotid glands of 2- and 24-month-old rats. The water soluble proteins of the glandular homogenate from the old and young rats are shown in lanes 1 and 3, respectively. The lanes 2 and 4 show the glycogen precipitate of the homogenate shown in lanes 1 and 3, respectively. The glycogen precipitate forms a single band with a molecular weight of about 56 000 as estimated by its electrophoretic mobility and retains the enzyme activity.

TABLE IV

PAROTID AMYLASE IN YOUNG AND OLD RATS
Precipitated as Amylase-glycogen complex.
The data represent mean ± standard error of means.

Age months	*Number rats*	*Number samples*	$\frac{\text{Dye Units}}{\text{mg DNA}} \times 10^{-6}$	$\frac{\text{Dye Units}}{\text{mg Amylase}} \times 10^{-6}$
2	8	16	37.1 ± 3.4	6.53 ± 0.46
24	6	13	44.7 ± 2.8	6.56 ± 0.33

*Dye units refer to the number of Cibachron Blue dye units released from the substrate of Cibachron Blue F3GA amylose complex. 1050 dye units = 1 mg of maltase produced from starch in 3 min incubation at 37°C (1 unit of amylase activity). The amylase was precipitated as the glycogen-enzyme complex from the glandular homogenate as described in the Materials and Method section.

and 18% of the total glandular proteins, respectively, in 2- and 24-month-old rats, maintains the enzyme activity (Table IV). The glandular contents of amylase in 2- and 24-month-old rats, calculated from the enzyme activity in the glycogen precipitate (Table IV) nearly equal those values which were obtained directly by the assays of the enzyme activity in the glands (Table I).

Despite the significant reduction in the rate of leucine incorporation into amylase and total proteins of the gland between 2 and 24 months, the glandular level of amylase does not appear to decrease during this period. There is no significant difference in the glandular contents of amylase at these ages, as indicated by the cellular level of amylase activity in the glands of 2- and 24-month-old rats (Tables I and IV). Furthermore, the specific activity of the enzyme is about the same in the glands of these rats (Table IV).

DISCUSSION

The decrease in the rate of incorporation of leucine into PCA-insoluble fractions in parotid glands between 2 and 24 months indicates that the rate of synthesis of total proteins declines with age and support our previous findings [6–8]. These earlier studies were done using the rats that had not been screened for the immunologic titers for SDA viruses. Therefore, the possibility existed that the reduced rate of protein synthesis reflected clinical manifestations of the viral infection rather than age-related, cellular changes. However, the results from this study, which employed only SDA negative rats, demonstrate that the age-related decline in the rate of protein synthesis is unrelated to the disease.

The results of the analysis of free leucine pool in the present study support that the difference in the rate of leucine incorporation into glandular proteins reflects the difference in the rate of synthesis of these proteins. There is no difference in the size of the intracellular leucine pool, but, there is about two times as much

radioactivity in this pool of the older rats than in the younger ones. This is the situation expected in the older rats with a reduced rate of protein synthesis. Thus, it is unlikely that the decrease in the rate of the amino acid incorporation in the older rats is due to the dilution of radioactivity due to the difference in the size of the precursor pool.

The rate of incorporation of leucine into the glycogen precipitate is also reduced in the older rats, indicating that the rate of amylase synthesis also declines with age. Several lines of evidence support that the glycogen precipitate is, in fact, amylase. The glycogen precipitate possesses the enzyme activity and forms a single band upon electrophoresis. The molecular weight of the glycogen precipitate, calculated from its electrophoretic mobility, is about 56 000 daltons which is the molecular weight of the parotid amylase [18]. Although the rate of incorporation of leucine into amylase in the older rats is somewhat more reduced than into the total protein (40% *vs.* 30%), it is uncertain whether this difference is significant.

The amylase, precipitated using glycogen, amounts to near equal proportions of total glandular proteins, 15% and 18% in young and old rats, respectively. Since the specific enzyme activity of amylase is about the same at these two ages, it is likely that glycogen precipitates the similar molar proportions of amylase in the glands of young and old rats. Furthermore, the glandular contents of amylase, determined from the glycogen precipitate, are about the same as those values obtained directly from the glandular homogenate. In the calculation of the total radioactivity in amylase or its enzyme activity, the values obtained from the glycogen precipitate were adjusted to account for the differences in the recovery of amylase by precipitation.

Since amylase is the major secretory protein of the parotid gland [19], the reduced rate of amylase synthesis may be extrapolated to the secretory proteins in general in this gland. One possible explanation for the reduced rate of secretory protein synthesis is that decremental changes occur in the cellular structure or mechanism involved in this process.

Various changes which affect the process of transcription and translation have been implicated. Although the DNA itself does not appear to change [22], alterations in the binding between DNA and nucleoproteins [22–25], and a shift in the nucleoprotein to a more basic type [26,27] have been reported. The changes in the nucleoprotein to a more basic protein repress parts of the gene expression and suppress protein synthesis. The changes implicated at the level of translation include the decrease in the amino acylation of tRNA [28–30], the activity of one of the elongation factors [31], the initiation process due to defects in a ribosomal subunit [32] and in the concentrations of polysomes [33] or active ribosomes [34]. Also, the decrease in the ribosome aggregation to mRNA related to reductions in the mRNA availability [1] have been reported. Whether any of these changes also occur during aging in the rat parotid glands needs to be determined.

However, it is also possible that the reduced rate of protein synthesis reflects the cellular regulatory mechanism of the synthesis of secretory proteins in relation to

the decrease in the level of digestive activity due to aging. It appears that amylase and other secretory proteins accumulate in parotid glands of old rats. We have shown previously that the number of degenerating secretory granules increases with age in the acinar cells of rat parotid glands, suggesting that secretory proteins remain in cells for a longer period of time in older animals than in young ones [20]. Also, as shown in this study, the glands of 24-month-old rats contain a relatively large amount of amylase, despite the decreased rate of protein synthesis, indicating that the level of secretory (discharge) activity is reduced in these old rats. On the other hand, the low content of amylase in the glands of 2-month-old rats, despite the high rate of protein synthesis, suggests a high level of secretory activity at this young age. These results support the suggestion that the cellular level of secretory proteins represents the balance between the rate of synthesis and secretion [1].

The decrease in the level of secretory activity is not due to the cellular changes which affect the process of discharge of secretory proteins [9]. Thus, the reduced level of secretory activity in the salivary gland is likely to be due to the reduction in the digestive and/or masticatory activities with age. The storage of secretory proteins in cells for a prolonged period of time, due to the reduced level of secretory activity, can affect the synthesis of more of these proteins by a positive feed-back type of inhibition [21].

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REFERENCES

1 A. Richardson, The relationship between aging and protein synthesis. In J.R. Florini (ed.), *Handbook of Biochemistry in Aging*, CRC Press, Inc., Boca Raton, Florida, 1981, pp. 79–101.
2 L. Hayflick, Cell Aging, In A. Cherkin et al. (eds.), *Physiology and Cell Biology of Aging* (*Aging*, vol. 8), Raven Press, N.Y. 1979, pp. 3–19.
3 J. Meyer and H. Necheles, Studies in old age. IV. The clinical significance of salivary, gastric and pancreatic secretion in the aged. *J. Am. Med. Assoc.*, *115* (1940) 2050–2053.
4 J. Meyer, J.S. Golden, N. Steiner and H. Necheles, The ptyalin content of human saliva in old age. *Am. J. Physiol.*, *119* (1937) 600–602.
5 R. Chilla, H. Niemann, C. Arglebe and G.F. Domagk, Age-dependent changes in the alpha-isoamylase pattern of human and rat parotid glands. *Oto-Rhino-Laryngology*, *36* (1974) 372–382.
6 S.K. Kim, P.A. Weinhold, S.S. Han and D.J. Wagner, Age-related decline in protein synthesis in the rat parotid gland. *Exp. Gerontol.*, *15* (1980) 71–85.
7 S.K. Kim, Age-related changes in the cellular level of amylase and protein synthesis in the rat parotid gland. *J. Dent. Res.*, *60* (1981) 738–749.
8 S.K. Kim, P.A. Weinhold, D.W. Calkins and V.W. Hartog, Comparative studies of the age-related changes in protein synthesis in the rat pancreas and parotid gland. *Exp. Gerontol.*, *16* (1981) 91–100.
9 S.K. Kim, D.W. Calkins and P.A. Weinhold, Secretion of α-amylase from parotid lobules of young and old rats. *Exp. Gerontol.*, *17* (1983) 387–397.
10 S.K. Kim, D.W. Calkins, P.A. Weinhold and S.S. Han, The changes in the synthesis of exportable

and nonexportable proteins in parotid glands during aging. *Mech. Ageing Dev., 18* (1982) 239–250.
11 S.K. Kim and D.W. Calkins, Secretory protein synthesis in parotid glands of young and old rats. *Arch. Oral Biol., 28* (1983) 1–4.
12 A.M. Jonas, J. Craft, C.L. Black, P.N. Bhatt and D. Hilding, Sialodacryoadenitis in the rat. A light and electron microscopic study. *Arch. Pathology 88* (1969) 613–622.
13 A. Loyter and M. Schramm, The glycogen-amylase complex as a means of obtaining highly purified α-amylases. *Biochim. Biophys. Acta, 65* (1962) 201–206.
14 K. Burton, A study of the conditions and mechanism of diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J., 162* (1956) 315–323.
15 P. Böhlen and M. Mellet, Automated flurometric amino acid analysis: The determination of proline and hydroxyproline. *Anal. Biochem., 94* (1979) 313–321.
16 K. Weber and M. Osborn, The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem., 244* (1969) 4406–6612.
17 T.L. Brown and F. Wold, Detection of α-amylase activity in unprocessed preamylase produced in the cell-free translation of porcine pancreatic RNA. *J. Biol. Chem., 256* (1981) 10743–10746.
18 M. Gorecki and E.P. Zeelon, Cell-free synthesis of rat parotid preamylase. *J. Biol. Chem., 254* (1979) 525–529.
19 D. Wallach, R. Tessler and M. Schramm, The proteins of the content of the secretory granules of the rat parotid gland. *Biochim. Biophys. Acta, 382* (1975) 552–564.
20 S.K. Kim, Changes in the secretory cells of the rat partoid gland during aging. *Anat. Rec., 209* (1984) 345–354.
21 J.H. Lillie and S.S. Han, Secretory protein synthesis in the stimulated rat parotid gland. *J. Cell Biol., 59* (1973) 708–721.
22 M.J. Phytila and F.G. Sherman: Age-associated studies on thermal stability and template effectiveness of DNA and nucleoproteins from beef thymus. *Biochem. Biophys. Res. Commun., 31* (1968) 340–344.
23 H.P. von Hahn, Stabilization of DNA structure by histones against thermal denaturation. *Experentia, 21* (1965) 90–91.
24 J.P. von Hahn and E. Fritz, Age-related alterations in the structure of DNA 3. Thermal stability of rat liver DNA related to age, histone content and ionic strength. *Gerontologia (Basel), 12* (1966) 237–250.
25 D.K. Kurtz and F.M. Sinex, Age-related differences in the association of brain DNA and nuclear protein. *Biochim. Biophys. Acta, 145* (1967) 840–842.
26 H.P. von Hahn, J. Miller and G.L. Eichhorn, Age-related alterations in the structure of nucleoprotein. IV. Changes in the composition of whole histone from rat liver. *Gerontologia (Basel), 15* (1969) 293–301.
27 A.R. O'Meara and R.L. Herrmann, A modified mouse liver chromatin preparation displaying age-related differences in salt dissociation and template ability. *Biochim. Biophys. Acta, 269* (1972) 419–427.
28 D. Mariotti and R. Ruscitto, Age-related changes of accuracy and efficiency of protein synthesis machinery in rat. *Biochim. Biophys. Acta, 475* (1977) 96–102.
29 L.L. Mays, A.E. Lawrence, R.W. Ho and S. Ackley, Age-related changes in function of transfer ribonucleic acid of rat liver. *Fed. Proc., 38* (1979) 1984–1988.
30 C.J. Wust and L. Rosen, Aminoacylation and methylation of tRNA as a function of age in the rat. *Exp. Gerontol., 7* (1972) 331–343.
31 K. Moldave, J. Harris, W. Sabo and I. Sadnik, Protein synthesis and aging: studies with cell-free mammalian systems. *Fed. Proc., 38* (1979) 1979–1983.
32 R. Comolli, Deficiency in accessory protein of native 40S ribosomal subunits in the liver of aging rats. *Exp. Gerontol., 10* (1975) 31–36.
33 U. Srivastava, Polyribosome concentration of mouse skeletal muscle as a function of age. *Arch. Biochem. Biophys. 130* (1969) 129–139.
34 D.I. Kurtz, A decrease in the number of active mouse liver ribosomes during aging. Exp. Gerontol., *13* (1978) 397–402.
35 D.K. Layman, G.A. Ricca and A. Richardson, The effect of age on protein synthesis and ribosome aggregation to messenger RNA in rat liver. *Arch. Biochem. Biophys. 173* (1976) 246–254.