FINSKA KEMISTSAMFUNDETS MEDDELANDEN

SUOMEN KEMISTISEURAN TIEDONANTOJA

REDAKTÖR – TOIMITTAJA
Gösta Brunow

INNEHÅLL – SISÄLTÖ

N:o 3 • ÅRGÅNG 81 VUOSIK. • 1972

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81 årg. 1972 N:o 3 81 vuosik.

Utgiven av — Julkaisija — Publisher
Finska Kemistsamfundet — Suomen Kemistiseura — Chemical Society of Finland
Postbox 10476 Postilokero
Helsingfors — Helsinki

CONTENTS

Isolation and Partial Characterization of Age-correlated Oligo Deoxyribo-ribo-nucleo-peptides*

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Summary

Oligo-deoxyribo-ribo-nucleo-peptides with comparative resistance to alkali and acid treatments, have been isolated from the organs of rats of different age levels. A correlation between the quantitative aspect of their formation and the increase in their molecular size and the age of the animals has been established.

Introduction

Though many theories of aging have been presented, there is still a scarcity of rigorous biochemical evidence.

To explain the so far irreversible phenomenon of aging, we must explore the possibilities for irreparable damage to DNA. Using rats which received tritium through their mothers at birth, we have traced such damaged DNA molecules and isolated tritium labeled oligo-deoxyribo-ribo-nucleo-peptides.

Our methodology involved tritiating mother rats and tracing tritiated molecules accumulated in vital organs of their offspring at different age levels. In the present experiments we administered 40 m.c. of ^{3}H-acetate and 8 m.c. of^{3}H-lysine to two mother rats and examined the liver and brain of two of the littermates of the former, 621 days old (A) and 493 days old (B), respectively, and the liver of the 363 days old offspring (C) of the second mother. These organs were extracted with saline, acetone, chloroform: methanol, and digested with pronase, trypsin and pepsin to obtain very small quantities of insoluble substances which were then subjected to progressively drastic conditions of alkali and acid treatment.

Alkali treatment of these insoluble substances (2 mg, 25 mg, and 12 mg) from the three rats respectively yielded oligo-deoxyribo-ribo-nucleotides with covalently bound peptides containing mainly aspartic and glutamic acids and a number of neutral amino acids. The molecular size of these compounds increased with aging indicating the progression of DNA damage with age. The results of characterization of these compounds have been presented previously (1).

* A summary of the results and conclusions of this paper was presented at 14de NORDISKA KEMISTMÖTET, at Umeå, Sweden, on June 19, 1971.

The acid treatment of the alkali insoluble substances again gave us a series of age-correlated oligo-deoxyribo-ribo-nucleotides with covalently bound peptides, with mainly aspartic and glutamic acids, arginine, and lysine and a number of neutral amino acids. These results we now present in this paper.

METHODS

I. *Preparation of Animals*

In Case I, 40 mc of^{3}H-sodium acetate was administered to a pregnant Sprague-Dawley rat perinatally according to a schedule previously published (2):

10 mc 7 days pre-partum
10 mc post-partum
10 mc 2 days post-partum
5 mc 5 days post-partum
5 mc 6 days post-partum

Eight apparently healthy offspring were born. Male offspring A was sacrificed at 621 days, while male offspring B died of pneumonia at 493 days of age. The liver of offspring A and the brain of offspring B were removed for subsequent study.

In Case II, 8 mc of^{3}H-lysine was administered perinatally to another pregnant Sprague-Dawley rat according to a schedule identical with that used for administration of ^{3}H-tyrosine in a previous publication (3):

2 mc 7 days pre-partum
2 mc 1 day post-partum
2 mc 2 days post-partum
1 mc 5 days post-partum
1 mc 6 days post-partum

Female offspring rat C was sacrificed at 363 days of age and its liver removed for comparative study.

II. *Preliminary Extraction of the Tissue*

The liver of rat A (wet weight 21.75 g), the brain of rat B (wet weight 1.51 g), and the liver of rat C (wet weight 7.5 g) were homogenized for $1^1/_2$ hours in a Virtis Homogenizer with about 5.3 ml per gram of 0.9 % saline solution. Homogenates were then centrifuged at 28,000 G, the sediments blended at room temperature with 10 times v/w A.R. acetone, allowed to equilibrate overnight at 1°C, and centrifuged the next day at 28,000 G. The acetone-insoluble substances were then given two similar extractions with 4 ml per gram (wet weight of the starting material $CHCl_3$: MeOH (2 : 1) at room temperature. The mixtures were then centrifuged at 28,000 G, twice more extracted in the same manner, and re-centrifuged. The sediments were finally washed with acetone and dried.

III. *Treatment with Proteolytic Enzymes*

The materials remaining after $CHCl_3$: MeOH extractions were incubated for three days with pronase (B-grade) in about 10 ml of KH_2PO_4 Na_2HPO_4 pH 10 buffer at 34°C. The weight of pronase used was about 7 % of the weight of the dry materials. Two drops of 1 % $CaCl_2$ solution were also added to the buffer before incubation. At the end of the incubation, the mixtures were centrifuged at 28,000 G, the sediments washed with buffer and water, and finally with acetone, and dried. The acetone-dried sediments weighed about 195 mg from the liver of rat A, 56 mg from the brain of rat B, and 88 mg from the liver of rat C.

10 mg from the residue of rat A, 47 mg from that of rat B, and 85 mg from that of rat C were each suspended in 30 ml of 0.067 M NaH_2PO_4 Na_2HPO_4 pH 7.8 buffer and incubated with trypsin (20 % of the substrates) at 37°C for 24 hours. The mixtures were then centrifuged in the above manner, washed twice with buffer, and twice with water, each time centrifuging and removing the supernatant.

The sediments were then suspended in 30 ml each of 0.01N HCl and incubated with pepsin (in concentrations similar to those in the trypsin digestion) at 37°C for 24 hours. The incubation mixtures were then centrifuged again at 28,000 G. The sediments were then finally washed with acetone and di-ethyl ether and dried. The final weights of the sediments were: 2 mg from the liver of rat A, 25 mg from the brain of rat B, and 12 mg from the liver of rat C.

IV. *Fleck & Begg Steps of Degradative Procedure*

The above materials were then subjected to the degradation procedure of Fleck and Begg (1965) (4) which was a modified procedure of Schmidt and Tannhauser designed to extract pure RNA (more precisely the oligo-ribo-nucleotides of RNA) free from DNA and acid soluble peptides. The first step in this procedure was to remove the "free nucleotides" (or oligo-nucleotides) with cold 0.6N $HClO_4$; the second step was to extract the oligo-ribo-nucleotides of RNA by incubating the residue with 0.3N KOH at 37°C for 1 hour; and the third step was to continue to incubate the remaining residue with 0.3N KOH for 5 hours to obtain polypeptide materials. We duplicated all three steps using RNA (Core: Sigma) as a control. Whereas with the aged rat tissue samples insoluble materials remained after the third step, with RNA control nothing remained after the second step.

V. *Acid Hydrolysis*

A. The materials remaining insoluble after treatment with 0.3N KOH at 37°C for 5 hours were each heated with 5 ml of 1N HCl for 1 hour. These mixtures were then centrifuged and the supernatants lyophilized.

B. The sediments were subjected to the same treatment as above but for 16 hours. The mixtures were centrifuged and the supernatants lyophilized.

C. The sediments were then each subjected to heating with 1 ml of 46 % $HClO_4$ for 3 hours. At this stage no insoluble substances remained.

VI. *Gel Filtration*

The lyophilized substances were redissolved in 0.5 ml water and charged on Sephadex columns ranging from G-25 to G-200 for initial separation of oligo-nucleo-peptides from peptides according to the range of their molecular sizes. Fleck and Begg criteria of the ratios of their U.V. absorbance at 232 nm/260 nm have been taken as the basis for this separation (See Method VIII A). Starting with Seph. G-75, those oligo-nucleo-peptides which were eluted within the void volumes of the columns were further chromatographed on Seph. G-100, those eluted within the void volume of G-100 were chromatographed on G-150 and those eluted within the void volume of G-150 were chromatographed on G-200. Oligo-nucleo-peptides which were eluted outside the void volumes of G-75 columns were chromatographed on G-25 columns. Column sizes were 0.8 cm × 25 cm. In all cases water was used as eluent except in one case of Fig. 6 where 0.08 M Ammonium Carbonate was used in order to see if better resolution could be obtained.

VII. *DEAE-Cellulose Anion-Exchange Column Chromatography*

Fractions eluted within and beyond the void volumes of the respective Sephadex columns were suitably pooled and charged on DEAE-Cellulose anion exchange columns of 0.8 cm i.d. × 25 cm height. These columns were prepared according to the method of Staehelin (1961) (5). The fractions pooled on Sephadex columns were not concentrated in order to allow maximum dilution for maximum ionization at 1°C. Chromatography was performed with 0.08 M NH_4HCO_3 pH 8.6, 2 ml fractions were eluted. No gradient was applied as it was intended to separate free peptides from oligo-nucleo-peptides, but not to effect a resolution of the oligo-nucleotides themselves, as was the case with Staehelin. In order to standardize the columns, acid soluble peptides were prepared from pepsin in perchloric acid following the procedure of Fleck and Begg. Oligo-ribo-nucleotides obtained

from RNA (Core : Sigma) were used in admixture with the peptides so prepared. Peptides were eluted from the column in the first fifteen 2-ml fractions while oligo-ribo-nucleotides were not found even after 150 fractions at a flow rate of 6 ml/hr. Absorbance measurements of the fractions were taken at 260 nm and 232 nm on a Beckman DU-Spectro-photometer.

VIII. *Characterization of the Fractions Eluted on DEAE-Cellulose Anion Exchange Columns*

A. Initial characterization of the fractions utilized the guide lines provided by Fleck and Begg's criteria for acid soluble peptides and oligo-ribo-nucleotides of RNA. Those fractions whose ratios of absorbance at 232 nm and 260 nm were 2 or greater than 2 were classified as peptides, and those whose ratios were between 0.5 and 1.2 were classified as oligo-nucleotides with covalently bound peptides (oligo-nucleo-peptides). Fleck and Begg's criterion for pure oligo-nucleotides of RNA is that the same ratio be between 0.3 and 0.5. The fractions so characterized were pooled accordingly. They were then lyophilized and put back in solution in 1 ml water. Aliquot portions of these solutions were taken for the following assays (from B to F below).

B. Charcoal-Cellulose Column Chromatography

Aliquots of samples were adsorbed on activated charcoal, then mounted on cellulose columns (0.8 cm × 50 cm), and eluted with a solvent consisting of equal volumes 95 % ethanol and 1M NH/OH saturated with $Na_2B_4O_4$ according to the method of Klenow and Lichtler (1957) (6).

C. Paper Electrophoresis

Paper electrophoresis of the above substances was performed on Whatman 3 MM paper in a buffer of pH 3.5 (H_2) : Pyr : HOAC : 287 : 1 : 10) at 2500 V, 150 m.a. for 45 minutes. Spots too weak to be observed under U.V. light were stained by dipping the paper in ninhydrine (0.2 % in acetone) and were visibly observed after drying at 110°C for 10 minutes.

D. Paper Chromatography

Paper chromatography of the above substances was performed on Whatman Grade I paper using a solvent containing one-half 95 % ethanol and one-half 5 % ammonium acetate in distilled water according to the method of Thach and Sundararajan (1965) (7).

E. Determination of Composition

Ribose of the above substances was determined by treating 0.1 ml aliquots of the 1 ml solutions according to the method of Dische (1955) (8). Prior to reaction, however, the samples were hydrolysed with 0.1 ml portions of 0.2N HCl at 100°C for 1 hour. Authentic L-ribose was used as a standard in a concentration of 18.4 nM per 0.1 ml. Three standards – – 0.1 ml, 0.2 ml, 0.3 ml – – were taken to give an average O.D. differential between 665 nm and 565 nm of 0.076 per 0.1 ml. The standards and the aliquots were diluted to 0.5 ml with water before reacting with 1 ml of Dische's orcinol reagent.

Deoxy-ribose was determined by treating 0.1 ml aliquots of the samples by the diphenylamine reaction according to Dische (1968) (9) using authentic deoxy-ribose as standard. Deoxy-ribose standards were made in such dilution that an average 42 nM were represented in 0.1 ml solution with an average O.D. of about 0.050 at 595 nm, when reacted with 1 ml of Dische's diphenylamine reagent. Color was developed in boiling water in 10 minutes. Three deoxy-ribose standards – – 0.1 ml, 0.2 ml, 0.3 ml – – were used. Burton's reaction with acetaldehyde gave high blank readings.

Prior to reacting with diphenylamine reagent, the samples and the standards were heated with 0.1 ml of 1N $HClO_4$ for 20 minutes in boiling water. No appreciable amount of deoxy-ribose was lost in the standards. Phosphorus in 0.1 ml aliquots in samples was determined according to the method of Bartlett (1959) (10). The standards were of such dilution that 50 λ of NaH_2PO_4 H_2O solution contained 50 nM of phosphorus giving an O. D. reading of 0.025 at 830 nm. Three standards – – 50 λ, 100 λ and 150 λ – – were used. Molar ratios of the nucleic acid bases were calculated following the method of Wyatt (1951) (11) with slight modifications. Aliquots of the samples (from 0.1 ml to 0.4 ml) were diluted to 1 ml with distilled water. A standard was made containing 14 μg of DNA in 1 ml H_2O. To each of the tubes containing the samples and to the standard 2 ml of 70 % perchloric acid was added and heated for 2 hours at 100°C. Samples were then cooled and neutralized with 2 ml of 10N KOH and the supernatants were brought to pH of 1. Their absorbance at 260 nm was measured and mean molar extinction coefficient for bases was taken on average as 10,000. The O. D. readings for the samples ranged from 0.017 to 0.200, and for the control DNA samples 0.065, which represented about 77 % of the bases present in the control expressed as adenine. No correction was made on the final base ratios of the samples.

In order to determine the amino acids, 0.1 ml portions of the above samples were totally hydrolyzed (with 0.1 ml of con. HCl) in sealed tubes at 110°C for 24 hours. The acid was evaporated,

the residues dissolved in 0.1 ml H_2O and placed on Whatman Grade 1 paper for electrophoresis. The electrophoresis was conducted in pH 6.5 buffer (consisting of v/v/v. Pyr : HOAC : water:: 300 : 12 : 2700) at 2500 V, 150 m.a., for 45 minutes. Known quantities of authentic amino acids were used as standards for electrophoresis. The dried paper was dipped in 0.2 % ninhydrin solution in acetone, heated at 100°C for 10 minutes, and the spots cut and eluted in 50 % ethanol in water. Color readings were taken at 570 nm. A known quantity of DNA was used for total hydrolysis and electrophoresis to compare the electrophoretic mobilities of the DNA degradation products with the amino acids in question.

F. Measurement of Radioactivity

Tritium was measured with a Tricarb Liquid Scintillation Counter, Model 3365, using 0.1 ml of the samples mixed with 15 ml of "Diotol" cocktail (Berdick and Jackman Laboratories).

Results

By hydrolyzing with acid the alkali-insoluble residues according to three successive steps, namely (1) with 1N HCl at 100°C for 1 hour, (2) with 1N HCl at 100°C for 16 hours and (3) with 46 % $HClO_4$ at 100°C for 3 hours, mixtures of oligo-nucleo-peptides and peptides of varying molecular sizes ranging from below 5000 to above 200,000 were obtained. The crude oligo-nucleo-peptide peaks eluted on Sephadex columns were further purified on DEAE-cellulose anion exchange columns in order to separate any traces of oligo-peptide peaks that came along with them. The pure oligo-nucleo-peptide peaks that were obtained from DEAE-cellulose columns were then examined on charcoal-cellulose column chromatograph, paper electrophoresis and paper chromatography as per Methods VIII B, C and D respectively. These formed single peaks on charcoal-cellulose columns. On paper-electrophoresis they moved slightly from the origin towards the cathode as single spots. On paper-chromatogram they also moved slightly from the origin as single spots.

Fig. I shows the gel-filtration on Seph. G-200 of the substances obtained after treatment of the alkali insoluble substances from rats A, B, C with 1N HCl at 100°C for 1 hour. Peaks $XIIa'_{200}$, $XIIIa'_{200}$ and $XIVa'_{200}$ which were eluted within the void volumes of their respective columns indicate mixtures of oligo-nucleo-peptides and peptides of mol. wt. above 200,000 and peaks $XIIa''_{200}$, $XIIIa''_{200}$ and $XIVa''_{200}$ which were eluted outside the void volumes indicate mixtures of mol. wt. between 150,000 and 200,000.

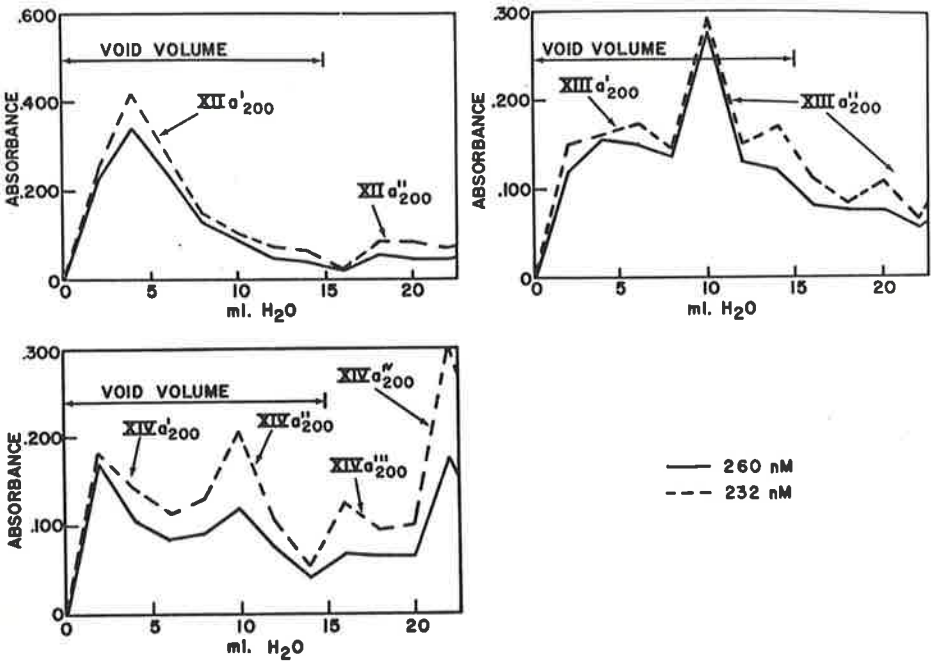

Fig. I- Gel-filtration on Seph. G-200 (0.8 × 25 cm) of the substances obtained from the alkali insoluble materials after treatment with 1N HCl at 100°C for 1 hour. Peaks $XIIa'_{200}$, $XIIIa'_{200}$ and $XIVa'_{200}$ represent mixtures of oligo-nucleo-peptides and peptides from rat A, B, C, respectively (mol. wt. above 200,000). Their $\frac{A232}{A260}$ ratio is about 1.2. The remaining peaks have mol. wts. ranging between 150,000 and 200,000.

Fig. II shows the DEAE-cellulose column chromatography of the substances eluted on Seph. G-200 column in Fig. 1. We note that in both the high and low molecular ranges (above 200,000 and between 200,000 and 150,000) these mictures were resolved into pure oligo-nucleo-peptides and oligo-peptides.' Quantitatively rat C revealed only about $^1/_4$ as much oligo-nucleo-peptide material as rat A, even though about 6 times as much insoluble material (insoluble after pepsin digestion) was used in rat C as in rat A.

Fig. III shows the gel-filtration on Seph. G-75 of the substances obtained by treatment with 1N HCl at 100°C for 16 hours. We find pure oligo-nucleo-peptide peaks in the void volume from rat A (XVa). From rats B and C, the oligo-nucleo-peptide peaks XVIc and XVIIc) occur only outside the void volumes. These

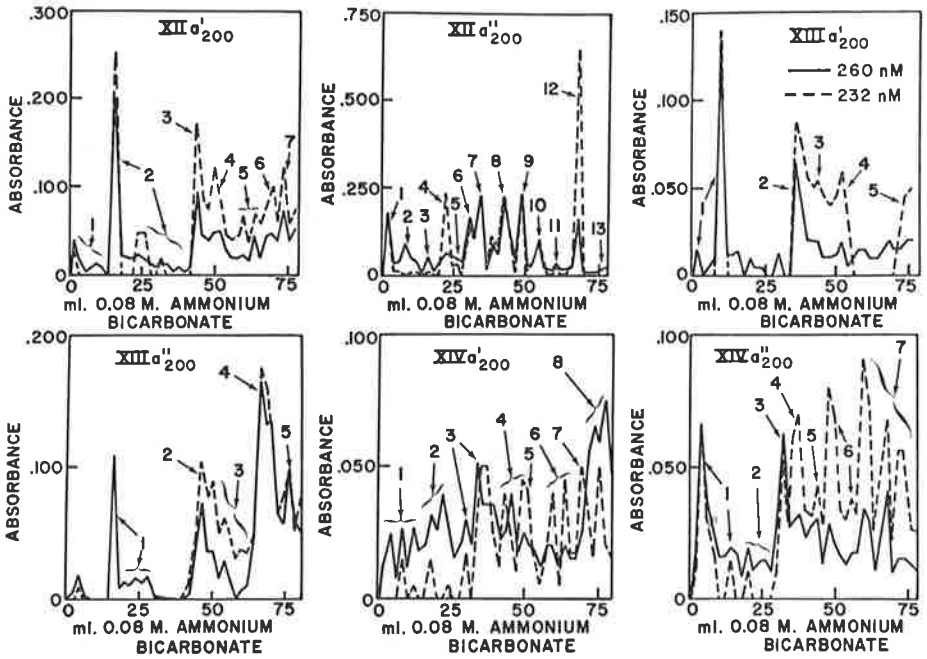

Fig. II- DEAE-cellulose anion exchange column chromatography of the substances obtained from rats A, B, C after treatment with 1N HCl at 100°C for 1 hour and gel. filtered on Seph. G-200 (0.8 × 25 cm). This separated oligo-nucleo-peptides from peptides. The $\frac{A232}{A260}$ ratio of the latter is about 2 or more.

low molecular size substances were further gel-filtered on Seph. G-25 (See Fig. IV).

Fig. IV shows the gel-filtration on Seph. G-25 of the low molecular substances (from Fig. III). From rat B we see at least two peaks $XVIc_a$ and $XVIc_b$ occuring within the void volumes. These indicate their molecular range to be above 5000. From rat C all of these substances fall outside the void volume ($XVIIc_c$).

Fig. V shows the DEAE-cellulose anion exchange chromatography of the substances XVa in Fig. III and $XVIc_c$ and $XVLIc_c$ in Fig. IV. The pure oligo-nucleo-peptide peaks, for example, from rat B ($XVIc_c$) are more numerous than those from rat C($XVIIc_c$). The latter are less than from rat A (XVa) in addition to being lower in molecular size.

Fig. VI shows Seph. G-75 gel filtration of the materials obtained after treatment with 46 % $HClO_4$ at 100°C for 3 hours. Rat A yielded crude oligo-nucleo-peptide peaks both in and

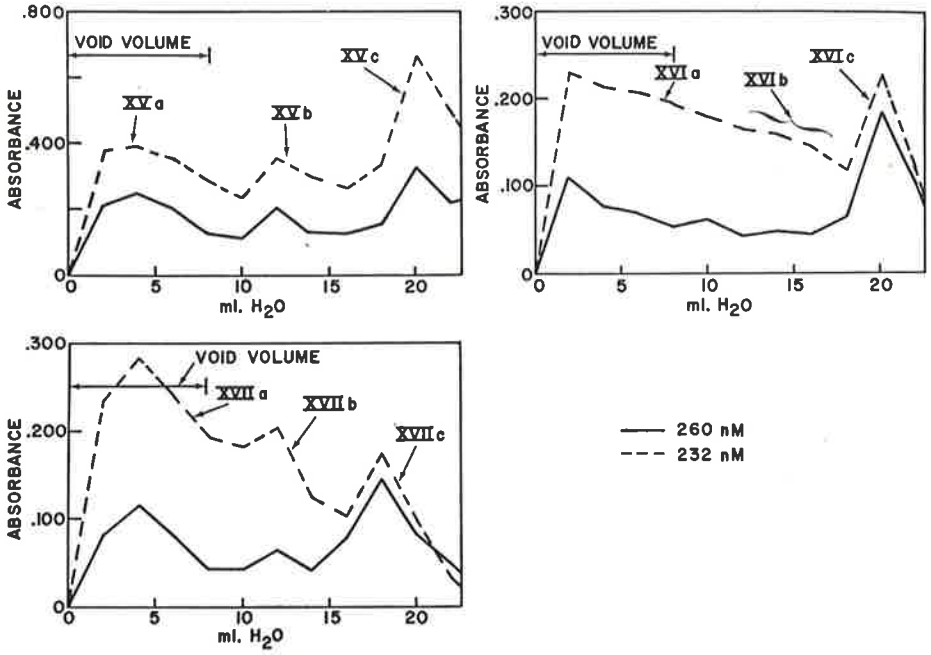

Fig. III- Gel-filtration on Seph. G-75 (0.8 × 25 cm) of the substances obtained from the insolubles (remaining insoluble after treatment with 1N HCl, 100°C, 1 hour) after treatment with 1N HCl at 100°C for 16 hours. XV, XVI, XVII represent the fractions from three rats A, B, c. Only rat A yielded the oligo-nucleo-peptide peak within the void volume. Rats B and C yielded oligo-nucleo-peptide peaks outside the void volumes. In their cases only peptides eluted within the void volumes with $\frac{A232}{A260} = 2$ or more.

outside the void volume of the respective column (XVIIIa, XVIIIb and XVIIIc respectively. Rats B and C on the other hand yielded these substances only outside the void volumes of the respective columns, XIXb, XIXc, and XXc.

Fig. VII shows the gel-filtration of Seph. G-25 of those fractions which came outside the void volumes in Fig. VI. Whereas rats A and B gave pure oligo-nucleo-peptide peaks both within the void volumes $XVIIIc_1$ and $XIXc_1$ and outside the void volumes XVIIIc/ and $XIXc_2$, rat C gave virtually no clear peak of oligo-nucleo-peptide.

Fig. VIII shows the DEAE-cellulose chromatography of some of the fractions of crude oligo-nucleo-peptides obtained in Fig. VII($XVIIIc_2$, $XIXc_2$, XXc_2). Whereas the rats A and B yielded

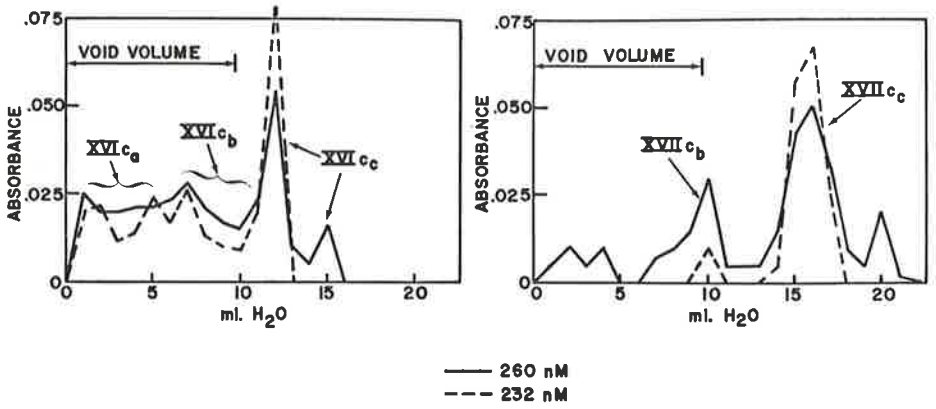

Fig. IV- Gel-filtration on Seph. G-25 (0.8 × 25 cm) of the oligo-nucleo-peptides eluting outside the void volumes of rats B and C in Fig. III.

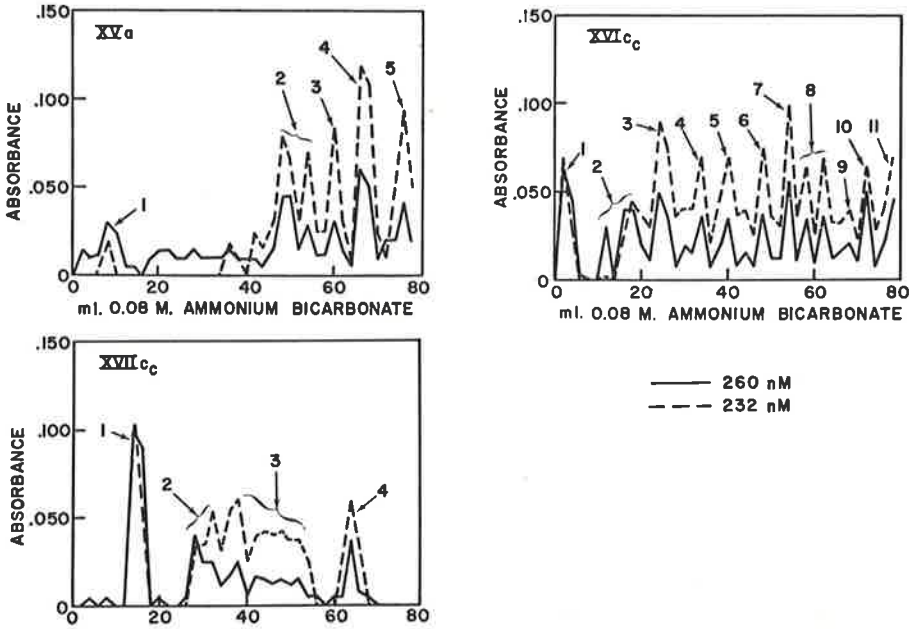

Fig. V- DEAE-cellulose anion exchange column chromatography of the substances obtained by gel-filtration (Fig. IV, III) to resolve them into pure oligo-nucleo-peptides and peptides.

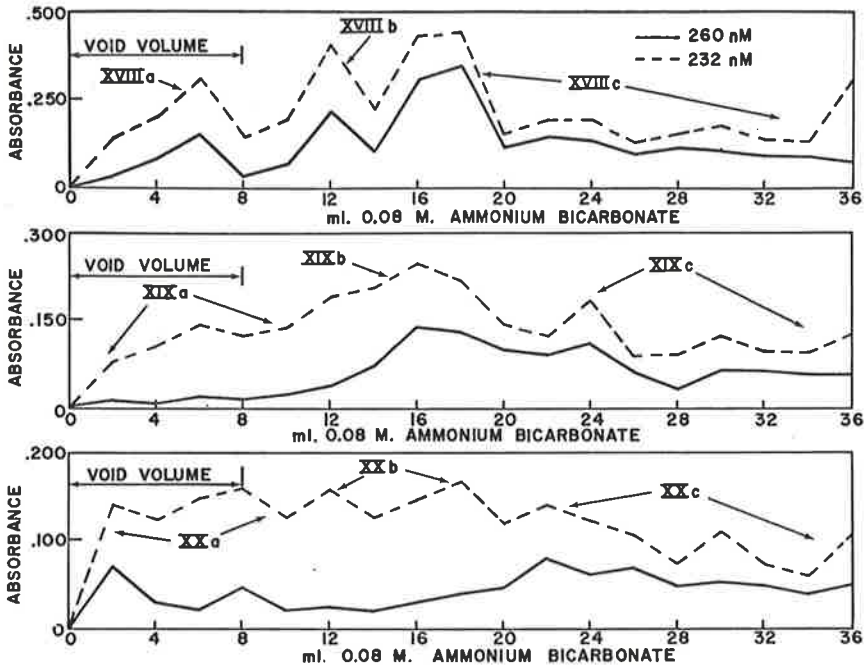

Fig. VI- Gel-filtration on Seph. G-75 (0.8 × 25 cm) of the substances obtained from the insolubles (remaining insoluble after treatment with 1N HCl at 100°C for 16-hours) by treatment with 46 % $HClO_4$ at 100°C for 3 hours from rats A, B, and C. All the crude oligo-nucleotide fractions eluted outside the void volumes of their respective columns.

both pure oligo-nucleo-peptides XVIIIc_{2-1} and XIXc_{2-1} and peptides XVIIIc_{2-2}, XVIIIc_{2-3}, XVIIIc_{2-4}, XVIIIc_{2-4}, XIXc_{2-4}, XIXc_{2-5}, XIXc_{2-6}, XIXc_{2-7} and XIXc_{2-8}; rat C yields virtually only oligopeptides XXc_{2-2}, XXc_{2-3}, XXc_{2-4}, XXc_{2-5}, XXc_{2-6}, and XXc_{2-7}.

Fig. IX shows the analysis of the oligo-nucleo-peptides obtained by treatment with 1N HCl at 100°C for 1 hour. We see in this table at a glance (a) that the molar ratios of total sugars to bases and phosphorus to bases vary widely from unity, (b) that in some high molecular fractions having molecular weights higher than 200,000 the content of deoxyribose is higher than that of ribose, but in some others the opposite is true, (c) that the bases far outnumber total sugars, (d) that the phosphorus ratios have drastically fallen and (e) that in the organs of all the rats, there is the predominance of ASP and GLU over neutral amino acids.

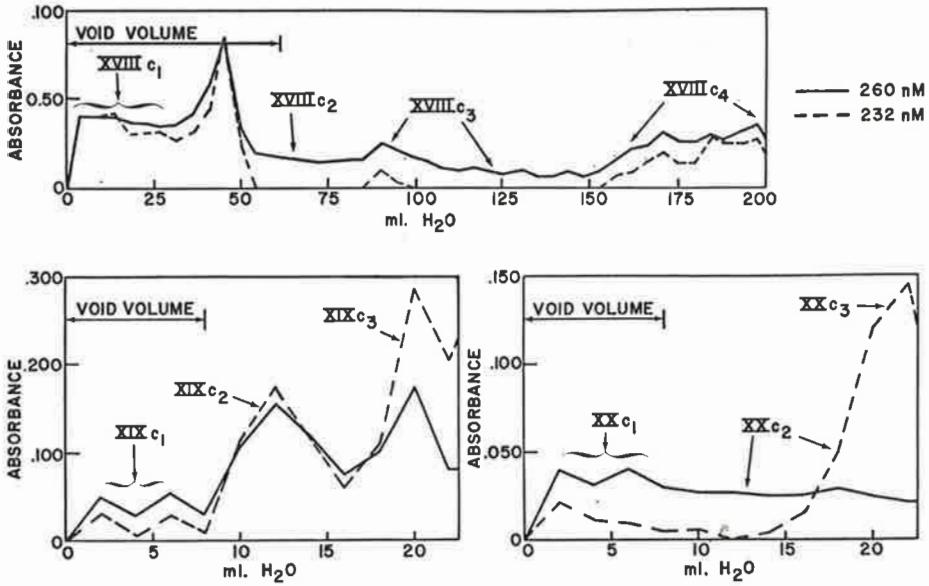

Fig. VII- Gel-filtration on Seph. G-25 (0.8 × 25 cm) of the crude oligo-nucleo-peptides obtained outside the void volumes of their respective G-75 columns (Fig. VI). Rats A and B yielded higher quantities of oligo-nucleo-peptides within their void volumes than rat C. The latter yielded only large quantities of peptides.

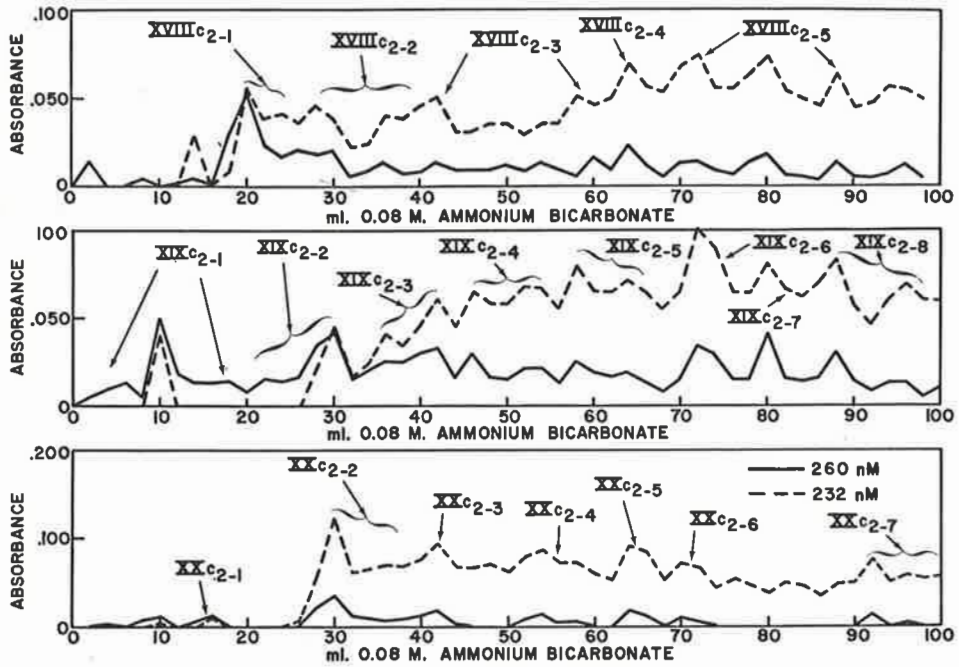

Fig. VIII- DEAE-cellulose anion exchange column chromatography of some of the substances obtained by gel-filtration on Seph. G-25 (Fig. VII). Note that rats A and B yielded only two oligo-nucleo-peptide peaks and that their peptide resolution patterns are similar. Rat C yielded only peptides in this case.

These observations lead us to the conclusion that the oligo-nucleo-peptide fractions we have are the products of original deoxyribo-ribo-nucleo-proteins which have undergone extensive structural damage involving cleavage of 3′, 5′-phosphodiester bonds and destruction of pentose and deoxypentose ring structures. If they have at least partially survived such drastic conditions of acid hydrolysis, it is because they are engulfed in denatured cytoplasmic proteins which we did not initially separate from the nuclei. The same feature of dephosphorylation persists in Fig. X (analysis of the oligo-nucleo-peptide fractions obtained by the treatment with 1N HCl at 100°C for 16 hours) and in Fig. XI (analysis of oligo-nucleo-peptide fractions obtained by the treatment with 46 % $HClO_4$ at 100°C for 3 hours. In Fig. XI, in the peptide part we see in some fractions relatively large quantities of ARG and LYS in addition to ASP, GLU, and neutral amino acids. At any rate the analyses do not throw any light as to the precise nature of the damage done to DNA due to aging but it does show that some damage is done due to its covalent bonding through peptides to RNA. Nor are we in a position to say anything about the exact linkages involved. The only result that can be related to aging is the appearance of a greater number of oligo-deoxyribo-ribo-nucleo-peptides in greater quantities and in greater molecular size in the fractions isolated from older rats than from the younger rat.

		Obtained by treatment of alkali insoluble residues with 1N HCl at 100°C for 1 hr.	Range of mol. weight	Ribose	Deoxy-ribose	Bases as adenine	Phos-phorus	Asp.	Glu.	His.	Arg.	Lys.	Neutral amino-acids as Ser.	Tritium DPM.
				expressed in nM										
1.	XIIa'$_{200-2}$	From liver of 621 day old rat (A)	above 200,000	156	198	1168	50	31.2	24	0	0	17.6	58.5	-
2.	XIIa'$_{200-3}$	"	"	116	6.2	195	75	21.9	10.5	0	0	0	12.5	-
3.	XIIa"$_{200-2}$	"	betw. 150,000 ~ 200,000	146	0	83	25	8.1	9.0				7.6	100
4.	XIIa"$_{200-8}$	"	"	46	53	174	75	9.2	7.5				12.5	
5.	XIIIa'$_{200-1}$	From brain of 493 day old rat (B)	above 200,000	24	394	1212	25	0	0	0	0	0	17.5	
6.	XIIIa'$_{200-2}$	"	"	138	16	171	50	5.8	13.5	0	0	0	7.5	
7.	XIIIa"$_{200-1}$	"	betw. 150,000 ~ 200,000	18	47	530	50	6.2	10.5				6.5	55
8.	XIIIa"$_{200-3}$	"	"	150	31	177	-	41.6	18			37.8	12.7	140
9.	XIVa'$_{200-1}$	From liver of 363 day old rat (C)	above 200,000	108	188	428	-	9.2	12				5	-
10.	XIVa'$_{200-5}$	"	"	8	70	70	-	78.9	52.5	0	0	0	12.5	95
11.	XIVa"$_{200-1}$	"	betw. 150,000 ~ 200,000	38	90	250	-	46.2	49.5	0	36.8	24.3	15	-
12.	XIVa"$_{200-3}$	"	"	20	70	89	-	0	0	0	0	0	4.3	-

Fig. IX- Analysis of oligo-nucleo-peptides obtained by treatment with 1N HCl at 100°C for 1 hour and purified on DEAE-cellulose columns.

		Obtained by treatment of alkali insoluble residues with 1N HCl at 100°C for 16 hrs.	Range of mol. weight	Ribose	Deoxy-ribose	Bases as adenine	Phos-phorus	Asp.	Glu.	His.	Arg.	Lys.	Neutral amino-acids as Ser.	Tritium DPM.
				expressed in nM										
13.	XVa-1	From liver of 621 day old rat (A)	above 50,000	66	112	318	-	6.9	19.5	0	0	0	7.5	100
14.	XVa-2	"	"	198	56	63	-	0	49.5	0	0	0	4.7	-
15.	XVIc$_{a-2}$	From brain of 493 day old rat (B)	betw. 5,000 ~ 50,000	90	90	174	-	0	7.5	0	0	0	3.7	25
16.	XVIc$_{a-5}$	"	"	31	126	88	50	0	10.5				7.5	135
17.	XVIc$_{b-1}$	"	"	158	154	346	50	0	6	0	0	0	9	55
18.	XVIc$_{b-8}$	"	"	32	146	83	50	0	13.5	0	0	0	4.3	30
19.	XVIc$_{c-2}$	"	below 5,000	52	62	67	-	0	12.0	0	0	0	1.3	-
20.	XVIc$_{c-6}$	"	"	38	112	48	-	0	13.5	0	0	0	3.3	-
21.	XVIIc$_{b-1}$	From liver of 363 day old rat (C)	about 5,000	25	169	63	-		6				2	-
22.	XVIIc$_{b-7}$	"	"	54	70	60	-		6				3.3	-
23.	XVIIc$_{c-2}$	"	below 5,000	0	83	110	50		15.8				10.6	80
24.	XVIIc$_{c-3}$	"	"	46	117	68	-		29.3				25.6	25

Fig. X- Analysis of oligo-nucleo-peptides obtained by treatment with 1N HCl at 100°C for 16 hours and purified on DEAE-cellulose columns.

	Obtained by treatment of alkali insoluble residues with 46% $HClO_4$ at 100°C for 3 hrs.	Range of mol. weight	expressed in nM										Neutral amino-acids as Ser.	Tritium DPM.
			Ribose	Deoxy-ribose	Bases as adenine	Phos-phorus	Asp.	Glu.	His.	Arg.	Lys.			
25.	$XVIIIc_{1-5}$ From liver of 621 day old rat (A)	betw. 5,000 ~50,000	29	182	79	250	46.8	36	0	0	0		8.6	-
26.	$XVIIIc_{1-8}$ "	"	88	96	80	50	12.1	22.5	0	22.6	0		20	95
27.	$XVIIIc_{2-1}$ "	below 5,000	56	104	66	-	7.7	15.8					7	10
28.	$XVIIIc_{3-1}$ "	"	96	143	139	-	12.1	11.3	0	38	0		12.6	80
29.	$XIXc_{1-1}$ From brain of 493 day old rat (B)	betw. 5,000 ~50,000	54	65	73	-	0	29.3		45	14.3		20	
30.	$XIXc_{2-1}$ "	below 5,000	127	57	211	100		29.3		58.7	24.3		20	
31.	XXc_{1-1A} From liver of 363 day old rat (C)	betw. 5,000 ~50,000	70	96	273	100	65.8	203.5		203.5	44.6		146.7	
32.	XXc_{1-4} "	"	35	32	88	50	13.9	11.3	0	0	0		10.6	

Fig. XI. Analysis of oligo-nucleo-peptides obtained by treatment with 46 % $HClO_4$ at 100°C for 3 hours and purified on DEAE-cellulose columns.

Conclusions

These results though preliminary indicate that:

(1) A correlation exists between aging of mammalian cells and the formation of compounds which appear to be oligo-deoxyribo-ribo-nucleo-peptides containing predominantly ASP, GLU, ARG, and LYS.

(2) Both the molecular size and the quantity of such compounds increase with age.

(3) Traces of tritium carried by some of these compounds show that they have remained fixed in the cells after surviving a lifetime of metabolic processes.

(4) These compounds are not merely broken single strands of DNA, and are not as such apparently susceptible to existing DNA repair mechanisms (thus making aging an irreversible phenomenon).

More work using more refined techniques is in progress.

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Acknowledgements

We wish to express our appreciation to the Glenn Foundation for Medical Research for a grant in partial support of this work. Our thanks are due to the various departments of the University of Wisconsin for making available to us their expertise and facilities in completing this work, and to Mr. Ted Shapas for assistance in the experimental work.

Received August 23, 1972

An INDO MO Study of the Anion Radical of Anisole.

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Abstract.

The total energy of the anisole anion radical as a function of the torsional angle has been calculated using the INDO approximation. The conformational behaviour of the methoxy group is found to be the same as in the neutral molecule. The coupling constant predicted for the methoxy group protons is of the same order of magnitude as the experimentally observed line width. Furthermore in agreement with the experimental results, the largest coupling constant is found for the protons in the meta position and the smallest for the proton in the para position. The calculated coupling constants and net charges show only small variations with the torsional angle.

Introduction

The anion radical of anisole has been studied several times by electron resonance spectroscopy. In the first of these by Nolfi Jr[1]., ESR studies of the cleavage and coupling reactions of a number of substituted anisoles were performed. At −110°C in a mixture of tetrahydrofuran and 1,2-dimethoxyethane (2 : 1 by volume) reduction with sodium-potassium alloy gave the anion radical of anisole. The electron resonance spectrum obtained was interpreted with the following coupling constants: $a_{ortho} = 5.34$ G, $a_{meta} = 6.06$ G and $a_{para} = 0.64$ G. This assignment was confirmed by Brown, Burnham and Rogers[2], who in addition studied the anion radical of the deutero compound 2,4,6-trideuteroanisole. The analysis of the electron resonance spectrum obtained from the deuterated compound clearly showed that the largest coupling constant should be attributed to the protons in the meta position. In a third study by Golubev, Khrolova and Grandberg [3] the electron resonance spectrum of the anion radical of anisole was reported. No assignment of the spectrum was given in that paper. The intensity ratios between the hyperfine components were reported however.

Brown *et al*[2] also performed molecular orbital calculations using the HMO method[4], the modified HMO method (ω-technique)[4] and McLachlan's approximate SCF method[5] in order to calculate the spin density in different parts of the molecule. The results of these calculations were disappointing in that they predicted either identical coupling constants for the ortho and meta protons (HMO) or a larger coupling constant for the ortho protons (the ω-technique and McLachlan's method) which is contrary to the experimental assignment based on the electron resonance spectrum of the deuterated radical. More recently Brown and

Burnham[6] reported π-electron UHF calculations on the anion radical of anisole, and found that this approximation gave a reasonable account of the spin density distribution in the radical, and also correctly placed the largest spin density in the meta positions.

Anisole is a typical example of an aromatic molecule with a saturated substituent. In HMO type π-electron calculations the influence of the methoxy substituent on the aromatic system must therefore be accounted for e.g. by an inductive parameter[4]. No satisfactory method to derive these parameters exists. In addition the planarity of the anisole molecule has not as yet been conclusively established[7], and in any case the methoxy group performs hindered reorientation (torsional oscillations) around the $C_{ar} - O$ bond. In nonplanar conformations it is not of course possible to make any simple σ-π separation. These facts make the parametrization of π-electron calculations difficult. To the knowledge of the present author no all valence electron calculations of the spin density distribution in anisole has appeared in the literature. In the present paper INDO MO calculations for different conformations of the anion radical of anisole are therefore reported.

Method

The INDO method (Intermediate Neglect of Differential Overlap), developed by Pople and coworkers,[8–10] was applied to the anion radical of anisole with the usual parametrization. The geometry of the molecule used in the present calculations, shown in figure 1, was obtained from electron diffraction data for anisole [11], and is discussed in ref. 7. However, the torsional angle between the plane of the aromatic ring and the plane containing the C_{ar}, O and C_{Me} is still open to choice. The equilibrium torsional angle is unknown both in the neutral molecule and in the anion radical. Because the calculated coupling constants perhaps depends on the torsional angle, the calculations were performed for a series of different torsional angles. For the different conformations all the various bond lengths and bond angles were kept constant, only the torsional angle was varied. The conformational behaviour of the methoxy group in the neutral anisole molecule has been studied previously using several different theoretical methods[7,12,13], and it has been observed that the rapid reorientation of the methyl group significantly influences the calculated torsional angle for which minimum in energy occurs[7]. The same two models, used also in ref. 7 giving minimum respectively maximum steric interaction between the methyl hydrogens and the ortho ring hydrogens were therefore used also in the present calculations. After annihilation of the

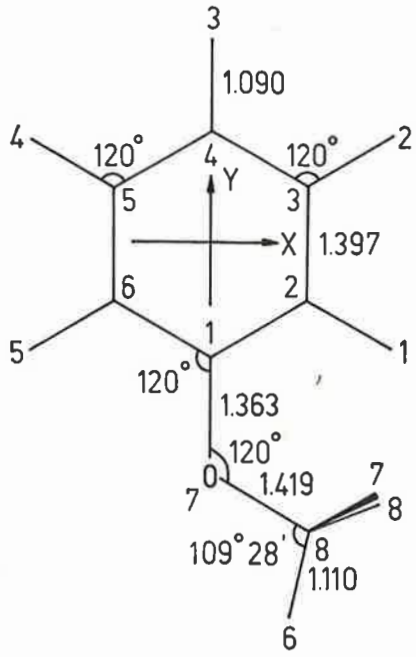

Figure 1. Bond lengths (Å) and bond angles in anisole.

quartet state contaminant, the proton hyperfine coupling constants were calculated according to the relation[14]:

$$a_H = 711.25\ \varrho_{S_H S_H}$$

where $\varrho_{S_H S_H}$ is the s-orbital spin density on the hydrogen atom under consideration. The computational details are given elsewhere [15].

Results and Discussion

Conformational Behaviour of the Methoxy Group.

The total energy of model A and model B of the anion radical of anisole for different torsional angles in shown in table 1, and a plot of the total energies versus torsional angle is shown in figure 2. It is seen that the conformational behaviour of the methoxy group in the anisole anion radical is exactly the same as in the neutral molecule. For model A the minimum energy is found for the planar conformation, for model B minimum in energy occurs for a torsional angle of 45°. The energy difference between model A and model B in the planar conformation is 0.223 eV (21.5 kJ/mole), and this difference can be taken as a

Table 1. The total energy of the anisole anion radical for different torsional angles and with two orientations for the methyl group. Model A corresponds to minimum, model B to maximum steric interaction between the methyl hydrogens and one ortho hydrogen in the aromatic ring.

Torsional angle	Total energy (eV)	
	Model A	Model B
0°	−1920.6372	−1920.4140
15°	−1920.6206	−1920.4702
45°	−1920.5795	−1920.5623
75°	−1920.5638	−1920.5357
90°	−1920.5604	−1920.5306

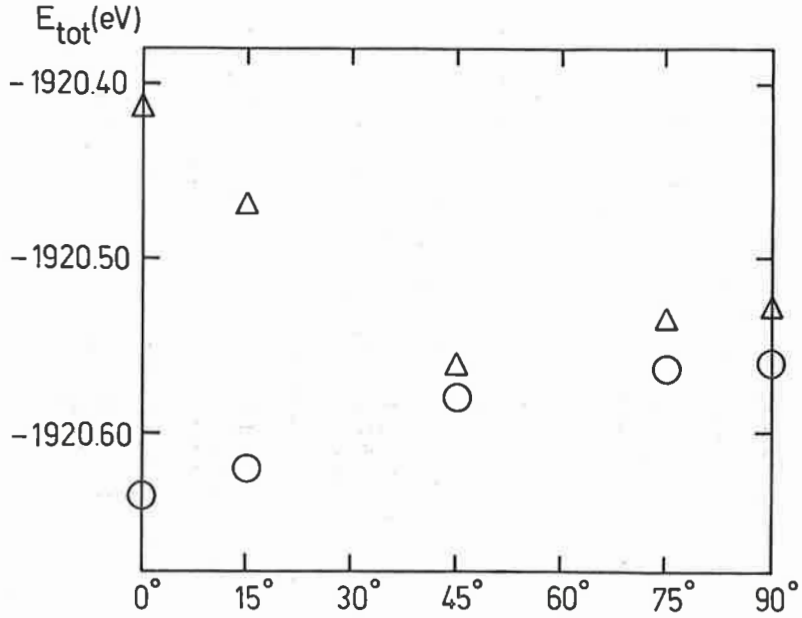

Figure 2. The total energy of the anisole anion radical as a function of torsional angle. O = model A, △ = model B.

measure of the torsional barrier[7]. This value is slightly larger than for the neutral molecule, indicating a small increase in the conjugation between the ring and the methoxy substituent caused by the unpaired electron in the anion radical.

Spin Density Distribution

The calculated hydrogen coupling constants for different torsional angles of the two models A and B are shown in table 2. The variations in the coupling constants are small and fall within the value of the observed line widths. It is also seen that the meta coupling constant is predicted to be the larger in agreement with the experimental assignment [1,2]. However, the magnitude of the calculated ortho and meta coupling constants is only about half the value observed experimentally, and the para coupling constant is nearly twice as large as the experimental value. The INDO approximation may therefore be considered to give the correct order among the coupling constants, and could be used as an aid in the assignment of measured coupling constants to definite positions in the molecule. However, the predicted magnitudes of the coupling constants are not reliable[15].

Table 2. The calculated proton hyperfine coupling constants in the anisole anion radical for different torsional angles. The expectation value of S^2 before annhilation 0.7870—0.7878, after annihilation 0.7505.

Model A

Nr	Torsional angle Θ				
	0°	15°	45°	75°	90°
1	−2.66	−2.68	−2.79	−2.77	−2.68
2	−3.03	−3.00	−2.84	−2.85	−2.94
3	0.97	0.97	0.93	0.91	0.91
4	−2.95	−2.97	−3.06	−3.03	−2.94
5	−2.72	−2.70	−2.56	−2.59	−2.68
6	0.02	0.05	0.10	0.01	0.01
7	−0.04	0.00	0.03	0.03	0.06
8	−0.04	−0.02	0.08	0.08	0.06

Model B

Nr	Torsional angle Θ				
	0°	15°	45°	75°	90°
1	−2.79	−2.78	−2.79	−2.78	−2.68
2	−2.92	−2.91	−2.84	−2.84	−2.94
3	0.96	0.95	0.93	0.91	0.91
4	−3.02	−3.02	−3.06	−3.04	−2.94
5	−2.60	−2.60	−2.55	−2.57	−2.68
6	0.04	−0.01	−0.02	0.09	0.10
7	0.04	−0.07	−0.08	0.00	0.04
8	0.04	0.14	0.11	0.07	0.04

Net Charges

The net charges in model A and model B of the anisole anion radical show only minor variations with the torsional angle. Negative net charges are found in the ortho and meta positions, the value at the ortho position being more negative. The results of the calculations of the net charges are given in table 3. In

Table 3. The atomic net charges of the anisole anion radical for different torsional angles.

Model A

Atom	Torsional angle Θ				
	0°	15°	45°	75°	90°
C_1	0.234	0.234	0.233	0.232	0.232
C_2	−0.188	−0.189	−0.191	−0.185	−0.180
C_3	−0.110	−0.109	−0.105	−0.107	−0.112
C_4	0.032	0.032	0.034	0.036	0.036
C_5	−0.108	−0.109	−0.115	−0.116	−0.112
C_6	−0.182	−0.180	−0.174	−0.174	−0.180
O_7	−0.301	−0.301	−0.304	−0.306	−0.306
C_8	0.267	0.266	0.266	0.268	0.269
H_1	−0.076	−0.076	−0.076	−0.077	−0.077
H_2	−0.094	−0.094	−0.094	−0.094	−0.093
H_3	−0.098	−0.098	−0.098	−0.098	−0.098
H_4	−0.093	−0.093	−0.093	−0.093	−0.093
H_5	−0.078	−0.078	−0.078	−0.077	−0.077
H_6	−0.079	−0.079	−0.076	−0.075	−0.075
H_7	−0.063	−0.065	−0.070	−0.069	−0.067
H_8	−0.063	−0.059	−0.058	−0.064	−0.067

Model B

Atom	Torsional angle Θ				
	0°	15°	45°	75°	90°
C_1	0.232	0.234	0.233	0.233	0.233
C_2	−0.196	−0.194	−0.190	−0.186	−0.180
C_3	−0.106	−0.106	−0.105	−0.107	−0.112
C_4	0.031	0.031	0.034	0.036	0.036
C_5	−0.112	−0.112	−0.116	−0.116	−0.112
C_6	−0.178	−0.177	−0.174	−0.175	−0.180
O_7	−0.299	−0.299	−0.303	−0.307	−0.307
C_8	0.247	0.251	0.264	0.269	0.270
H_1	−0.073	−0.074	−0.077	−0.077	−0.077
H_2	−0.095	−0.095	−0.094	−0.094	−0.093
H_3	−0.099	−0.099	−0.098	−0.098	−0.098
H_4	−0.093	−0.093	−0.093	−0.093	−0.093
H_5	−0.078	−0.078	−0.077	−0.077	−0.077
H_6	−0.031	−0.035	−0.051	−0.059	−0.060
H_7	−0.077	−0.078	−0.077	−0.075	−0.075
H_8	−0.077	−0.075	−0.074	−0.074	−0.075

the planar conformations the xy-plane is a symmetry plane and a separation of the molecular orbitals into one set of σ-electron molecular orbitals and one set of π-electron molecular orbitals may be performed. The set of π-electron molecular orbitals contains only $2p_z$ atomic orbitals and those hydrogen 1s atomic orbitals which have their centres symmetrically under and above the xy-plane. The π-electron densities may then be calculated as the total electron population of the atomic orbitals constituting these molecular orbitals. The result is given in table 4. It is

Table 4. π-electron densities in the planar conformation of the anisole anion radical.

Atom	C_1	C_2	C_3	C_4	C_5	C_6	O_7	C_8
Model A	0.952	1.304	1.247	1.003	1.244	1.309	1.902	0.918
Model B	0.950	1.313	1.241	1.004	1.250	1.302	1.902	0.898

seen that as far as the π-electron densities are concerned the results of the present calculations are very similar to the results of the π-electron UHF calculations given by Burnham[16].

The site of initial protonation in the Birch reduction of anisole is not conclusively known[2,16–20]. Calculations of the charge densities have been performed using different approximations[2,16,20] in order to gain insight into the problem. However, as pointed out by Burnham,[16] it is not obvious that protonation will occur at the site with the highest charge density. The energy of the transition state[16] and the difference in stability between the ortho and meta protonated products should probably play the most important role. The charge density and spin density at definite positions in the molecule is probably of less importance. More insight could be gained from charge density and spin density maps[21] giving the variation in these densities in regions of space surrounding the molecule.

Acknowledgements.

The author is indebted to Svenska Vetenskapliga Centralrådet for financial aid.

Received October 22, 1972.

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Litteratur

Terje Enkvist: *The history of Chemistry in Finland 1828—1918* (del 6 av The history of learning and science in Finland 1828—1918), 161 sidor, 42 bilder. Utgiven av Finska Vetenskapssocieteten. Häftad 30 mk.

Fröet till kemin i Finland kan sägas vara inrättandet av en lärostol i ämnet 1761 vid den gamla Åbo Akademi. Redan under universitetets Åbo-tid intog den kemiska forskningen i landet en även i utlandet respekterad ställning främst kanske genom Johan Gadolins arbeten. Kemin var alltså en etablerad betenskap när Universitetet efter Åbo brand överflyttades till Helsingfors 1828. Vad som sedan hände på kemins område fram till slutet av 1800-talet är väl för kemisten i dag ett *terra incognita*. Professor emeritus Terje Enkvists nyligen utkomna lärdomsvetenskapliga framställning av kemins utveckling och resultat från 1828 till 1918 är därför välkommen.

Enkvist framställning baserar sig främst på personhistoria, vilket kanske är förklarligt eftersom själva vetenskapens historia inte blivit lika fyllig och givit en betydligt sämre bild av utvecklingen. Med naturnödvändighet blir det därför främst kemisterna vid Helsingfors Universitet som skildras. Till följd av trenden under olika tider kommer likväl också de olika kemiområdenas att presenteras i tämligen sammanhängande avsnitt. Den oorganiska kemin övervägde under tidsperiodens första del (fram till ungefär 1850) genom Pehr Adolf Bonsdorff och Adolf Moberg. Den organiska kemin åter introducerades av Adolf Edvard Arppe och utvecklades under J. J. Chydenius och Edvard Hjelt för att med Ossian Aschan och Gustaf Komppa nå internationell nivå och erkännande. Den fysikaliska kemin kom naturligtvis långt senare och dess första företrädare på professorsnivå blev Lars W. Öholm. Axel Rindell var den första framstående läraren och forskaren inom agrikulturell kemi och biokemin företräddes av E. Sundvik och J. Hämäläinen. Bland mineralkemisterna märks A.B. af Schultén och Walter Wahl.

På den tillämpade kemins och teknologiens område verkade först driftiga apotekare såsom Erik och Johan Julin, G. A. Serlachius, J. Granberg m.fl.

Under nästan hela den skildrade epoken var de ekonomiska förhållandena små och outvecklade. De resurser som kunde ställas till vetenskapens disposition var därför starkt begränsade. För kemins del märkes detta t.ex. i en ständig kamp för tillräckliga och ändamålsenliga laboratorier.

Enkvists personhistoriska grepp har gjort att han tyckts sig tvungen att skildra kemisternas (främst professorernas) görande och låtande även utanför den kemiska ramen. Sålunda upptar Arppes verksamhet såsom rektor och censor och Hjelts såsom rektor och politiker rätt stora utrymmen. Om detta skall räknas som ett positivt eller negativt drag är väl en bedömningsfråga. Personligen skulle undertecknad gärna sett mer kemi. Å andra sidan kan man säga att Enkvist visat att kemister kunde användas utanför sina laboratorier.

Med tanke på eventuella utländska läsare skildrar Enkvist på ett utrymme som svarar mot c:a 30 % av hela boken den politiska och ekonomiska bakgrunden. En finländsk läsare kan gott hoppa över största delen av detta avsnitt.

Enkvists bok fyller utan tvivel ett behov. Då den dessutom är lättläst och stilen uppluckrad med en och annan anekdot eller roande detalj blir den inte tråkig och kan därför rekommenderas för var och en kemist, som vill veta var han står i ett historiskt sammanhang.

Gust.-Ad. Holmberg.

Frenckellska Tryckeri Ab, Helsingfors 1972