Biochimica et Biophysica Acta, 630 (1980) 447—458

BBA 29299

THE SOLUBLE COMPONENTS OF CHROMAFFIN GRANULES

A CARBON-13 NMR SURVEY

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(Received October 10th, 1979)

Key words: Chromaffin granule; ^{13}C-NMR; Catecholamine; Adenine nucleotide; Chromogranin

Summary

Carbon-13 NMR spectra of the reconcentrated chromaffin granule lysate have been obtained at 50 MHz and 62.9 MHz. The spectrum contains a number of assignable resonances in addition to those of the main soluble components (catecholamines, adenine nucleotides and chromogranin). Guanine and uridine nucleotides are present at levels of 0.13 and 0.08 mol/mol adenine nucleotides, respectively. Concentrations of cytidine nucleotides and NAD^+ are below the detection limit (0.02 mol/mol adenine nucleotides). An unidentified low molecular weight species, thought to be an adenine-containing oligonucleotide, is also present. Ascorbic acid was observed at a concentration of 0.14 mol/mol adenine nucleotides, but both dopamine and dehydroascorbic acid were below the detection limit. Protein resonances agree well with the reported amino acid composition of chromogranin A, with the exception of tryptophan and glutamine which have not previously been measured. The concentrations of these residues are estimated to be 12 ± 3 and 39 ± 5 residues per 77 000 dalton unit of chromogranin A. Substantial intensity due to unsaturated fatty acid side-chains in solubilized lipid is seen in the olefinic carbon region and in the methylene region, suggesting the presence of lipoprotein. Unassigned carbohydrate resonances are also present, but are largely obscured by sucrose in the isolation medium.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CNP, GNP, UDP, cytidine-, guanosine- and uridine-5'-nucleoside phosphates; DSS, 2,2-dimethyl-2-silapentane-S-sulfonate.

Introduction

It has been known for over two decades that the main soluble components of chromaffin granules are catecholamines (epinephrine plus norepinephrine), ATP and the protein chromogranin, which is present largely in random coil form [1—4]. More recently, a number of minor soluble components have also been reported. These include ascorbate (0.035 mol/mol epinephrine [5]), dopamine (0.011 mol/mol epinephrine [6]), dopamine-β-hydroxylase [7,8], divalent metal ions [9,10] and mono-, di- and triphosphonucleotides of adenine and guanine [11,12]. In a study of nucleotide composition, Goetz et al. [12] have reported that GDP and UDP comprise 6.9% of total nucleotides and, thus, uridine nucleotides are also possible soluble components. The composition of chromaffin granules has recently been reviewed critically by Winkler [13].

Carbon-13 NMR spectra provide an efficient method of surveying the soluble components of chromaffin granules. The spectra contain resonances of all carbon-containing compounds and thus are particularly useful in the identification of unexpected components. The main drawback of the technique is its relatively low sensitivity. Concentrations below the millimolar range require prohibitive accumulation times for confident identification.

We have previously published ^{13}C-NMR spectra of intact chromaffin granules at 25.03 MHz [14]. The present work reports spectra of the reconcentrated chromaffin granule lysate at 62.905 MHz. The latter spectra provide more than an order of magnitude sensitivity increase over the spectra of intact granules at 25 MHz and contain assignable resonances from species present at a relative concentration of 0.005 mol/mol catecholamine. Most of the established soluble components, except dopamine-β-hydroxylase, are visible at this level. The attainable spectral resolution is also approximately an order of magnitude higher than in previous studies. This results both from increased dispersion at higher field and from the marked decrease in intrinsic linewidth that accompanies lysis. In analysing the spectra, we have first assigned resonances as completely as possible in terms of the established soluble components listed by Winkler [13]. Unassigned resonances were then examined with a view to detection of unexpected species.

Experimental

Chromaffin granules were isolated from bovine adrenal medullas as described by Kirshner [15], except that they were sedimented and washed three times in an isolation medium containing 0.3 M sucrose, 10 mM Hepes (pH 7.0). The granules were lysed by osmotic shock using the procedure of Njus et al. [16]. After addition of 200 ml distilled water to the pellet, the membrane fraction was removed by centrifugation for 20 min at 39 000 × g. Centrifugation was repeated to assure removal of membrane fragments. The supernatant was lyophilized and yielded 708 mg solid. The solid was stored at −20°C for 2 days, then redissolved in $^{2}H_2O$ to a concentration of 345 mg residue/ml. The suspension was stored frozen for approx. 2 weeks until used.

Proton-decoupled ^{13}C-NMR spectra of the lysate were obtained at 62.905 MHz using a Bruker WH250 spectrometer. Spectra were obtained with and

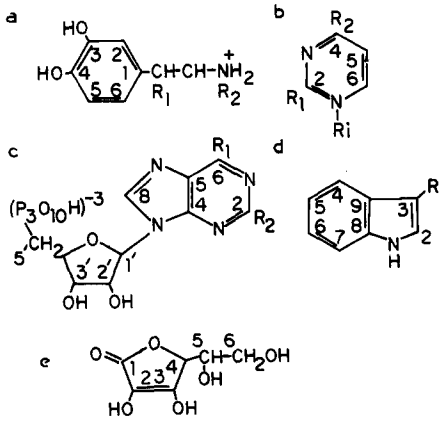

Fig. 1. Numbering systems used in the assignments of Figs. 2—5: a, catecholamines; b, pyrimidine nucleotides; c, purine nucleotides; d, tryptophan; e, ascorbic acid and dehydroascorbic acid.

without nuclear Overhauser enhancement at 3°C. Each spectrum required 10 000 transients (16K data points), with a 0.85 s delay and a 90° nutation angle. Partially relaxed spectra (5000 transients per point) were obtained at 10°C using a Varian XL-200 spectrometer.

^{13}C chemical shifts are expressed in ppm from internal DSS with positive shifts to low field. Assignments are based on the numbering system of Fig. 1. Assignments of epinephrine and sucrose were taken from Johnson and Jankowski [17]. The C-2 resonance of epinephrine was used as an internal reference and was assumed to lie at 114.8 ppm. Chemical shifts of norepinephrine, dopamine, ATP and ADP in ^2H_2O at p^{2}H 5.9—6.1 and 25°C were measured at 25.03 MHz on a JEOL JNM-PFT/100 spectrometer. Chemical shifts of ascorbic acid and dehydro-ascorbic acid at pH 5.5 were taken from the work of Berger [18]. Shifts of nucleotides other than ATP and ADP were taken from the tables of the report of Rosenthal and Fendler [19]. Assignments of amino acid resonances are generally the same as those used in a previous study [14], except that the shifts of histidine have been corrected to pH 5.6 using the data of Quirt et al. [20]. The tables of Rosenthal and Fendler [19] have been used extensively in assignments of lipid, nucleotide and carbohydrate resonances.

Monosodium ATP was purchased from Aldrich Chemical Co. and Na_3ADP (Sigma) was a gift from Dr. Charles Yocum. Dopamine hydrochloride was purchased from Sigma Chemical Co. and all other chemicals were reagent grade.

Results

After lyophilization, the solid residue of the chromaffin granule lysate was dissolved in ^2H_2O to a concentration of 345 mg/ml. The proton-decoupled ^{13}C spectrum (62.905 MHz) of this solution is shown in Fig. 2. The spectrum is dominated by sharp, intense resonances of sucrose, epinephrine and norepinephrine. Assignments of these and other peaks are described above. Figs. 3—5 are expansions of the spectrum in Fig. 2. A large number of

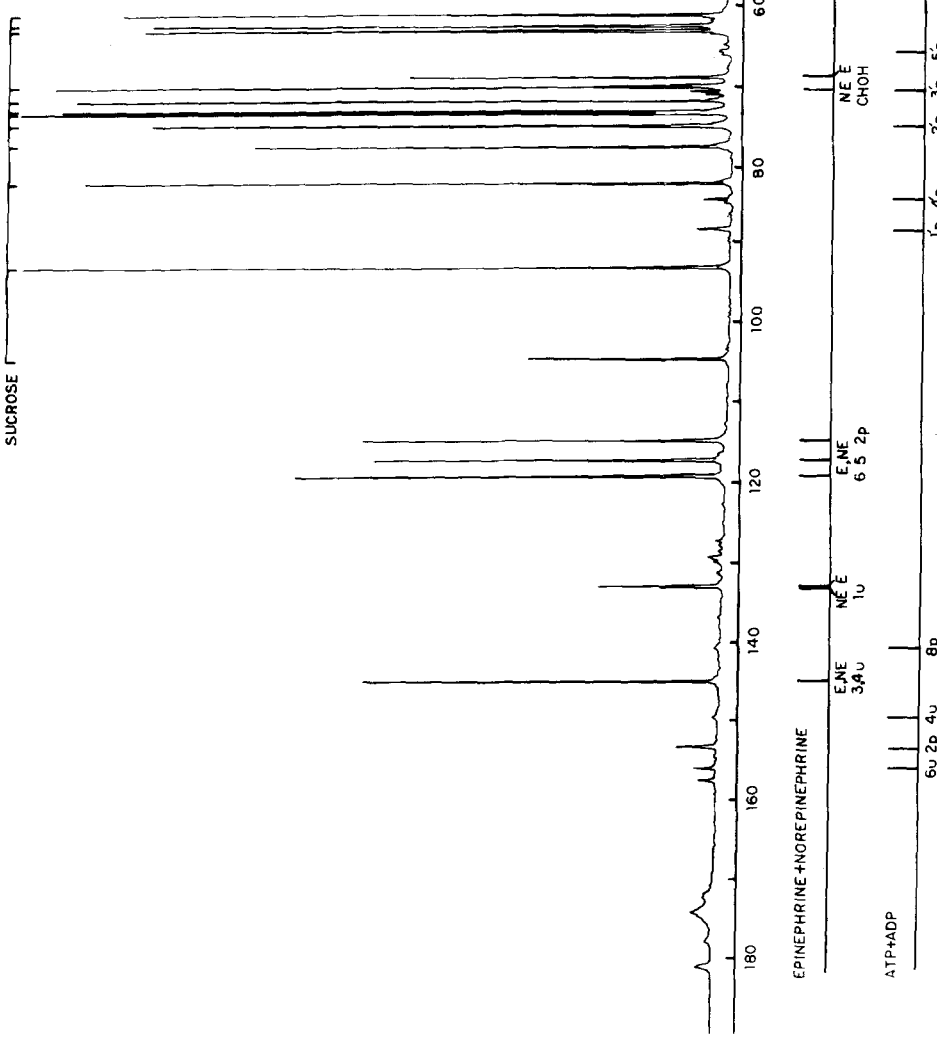

Fig. 2. ^{13}C-NMR *spectrum of the reconcentrated chromaffin granule lysate at* 62.905 MHz, p^{2}H 6.2, T = 10°C. Chemical shifts are expressed in ppm from internal DSS and were measured from the C(2) resonance of epinephrine, for which the shift was taken to be 114.8 ppm [17]. Sucrose resonances are indicated in the spectrum. Resonances of the Hepes buffer fall in the range 48–59 ppm, and are indicated in Fig. 5. Assignments of the predominant catecholamines and nucleotides correspond to the numbering system in Fig. 1; p and u specify protonated and unprotonated carbons. ATP and ADP resonances coincide at p^{2}H 6.0.

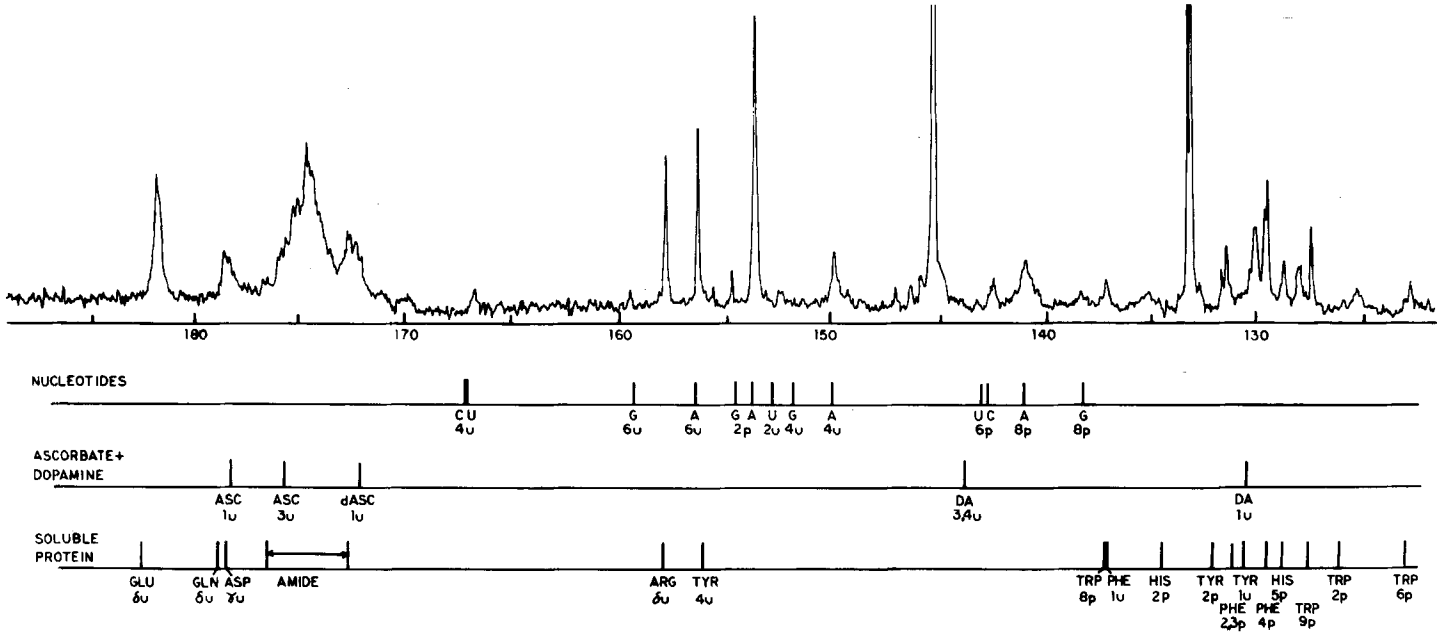

Fig. 3. Expansion of the downfield region of Fig. 2. Assignments are given for cytidine (C), uridine (U), guanine (G) and adenine (A) nucleotides, for absorbate (ASC) and dehydroascorate (dASC), for dopamine (DA) and for amino acid residues of the soluble protein. The numbering system is that of Fig. 1. Assigned carbon resonances are specified as protonated or unprotonated (p or u).

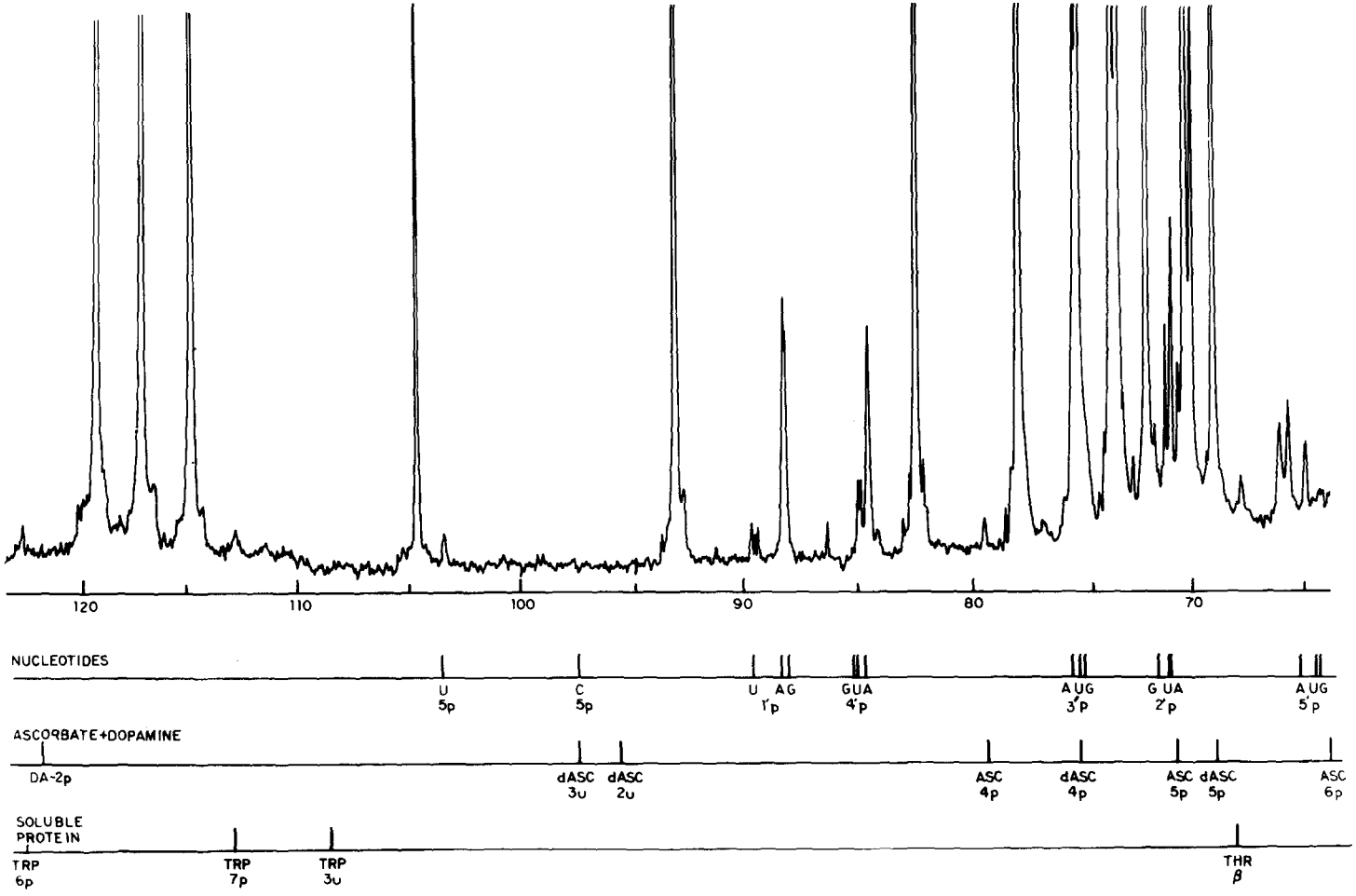

Fig. 4. Expansion of the central portion of the spectrum in Fig. 2. For assignments of catecholamine and sucrose resonances, and for experimental details, see Fig. 2.

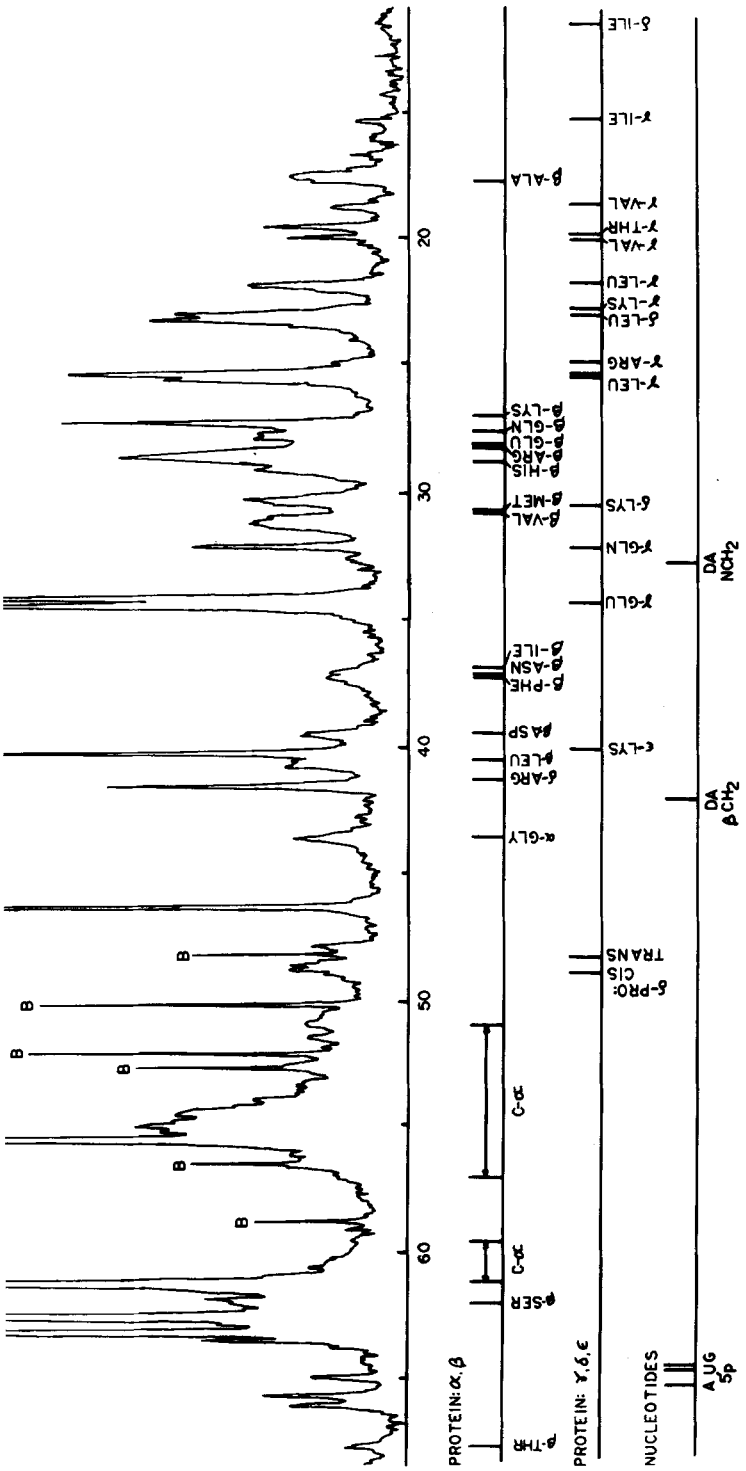

Fig. 5. Expansion of the upfield region of Fig. 1. Resonances of the Hepes buffer (B) are indicated. Assignments are given for amino acid residues of the soluble protein, for the 5'-nucleotide resonances and for dopamine. For assignments of major catecholamine and sucrose resonances, see Fig. 1.

resonances that had been unresolved or obscured by noise at 25.03 MHz are visible in these spectra. The detection limit is approx. 3 mM in the lysate or $5 \cdot 10^{-3}$ mol/mol catecholamine.

We have attempted to assign these spectra as completely as possible in terms of the established soluble components listed by Winkler [13]. These species comprise nucleotides, ascorbate, catecholamines and the soluble protein chromogranin (it is assumed that the resonances of soluble enzymes, such as dopamine-β-hydroxylase, are broad and do not contribute to the resolvable structure). In addition to these components, dehydroascorbate and the cytidine nucleotides, which were considered reasonable candidates as natural components of the lysate, are included. A detailed inspection of the spectra shows, in general, close agreement between predicted and observed resonances among the established components.

Nucleotides in chromaffin granules consist chiefly of adenine nucleotides which are present as ATP (70% of total nucleotides), ADP (11.3% of total nucleotides), and AMP (2.3% of total nucleotides) [12]. At pH 5.6 the resonances of ATP and ADP nearly coincide and are unresolvable, while AMP is essentially at the detection limit. One of the ATP + ADP ribose peaks, C-2, is obscured by sucrose and one of base peaks, C-5, is obscured by catecholamine. The remaining peaks are resolved and are fairly intense. Among the base resonances, C-8 shows pronounced broadening ($\Delta\nu_{1/2}$ = 40 Hz) and C-4, which is unprotonated and has lower integrated intensity than the protonated carbons, is broadened to a lesser degree. Selective broadening at these resonances is a characteristic effect of the binding of paramagnetic metal ions to the phosphate esters of adenine nucleotides. Broadening induced by Mn(II) and Cu(II) has been studied extensively [21—23] and has been shown to result from bridged structures in which the metal chelates both to the β and γ phosphate esters and to N7 on the purine ring. Broadening of the carbon resonances is in the order C-8 $\approx$ C-5 > C-4 > C-6, with little or no effect on C-2 and the ribose carbons. Evidently, fairly high concentrations (approx. 0.05 mM) of divalent paramagnetic ions are present in the lysate.

In addition to adenine nucleotides, guanidine and uridine nucleotides are present in the lysate and give resolvable peaks in both the base and ribose regions. The guanine base shows a well-defined protonated resonance due to C-2 at 154.8 ppm. Resonances of the unprotonated carbons have lower intensity, while that of the protonated carbon C-8 is broadened like that of the corresponding adenine resonance. Uridine nucleotides are present at a concentration comparable to that of guanidine nucleotides. The C-5 (protonated) resonance gives rise to a well-resolved peak at 103.5 ppm for which the assignment is unmistakable. The corresponding peak of cytidine nucleotides is not present, indicating a concentration below the detection level (0.02 mol/mol adenine nucleotides) for these species. Thus, intensity at 166.8 ppm and 142.8 ppm may be attributed to the C-4 (unprotonated) and C-6 (protonated) carbons of UNP without appreciable contribution from the corresponding CNP resonances.

Concentrations of guanidine and uridine nucleotides have been estimated from peak integrals (without nuclear Overhauser enhancement) of the C-2 (protonated) and C-5 (protonated) resonances of GNP and UNP, respectively. Measured integrals of these peaks were compared to the integral of the well-

TABLE I

ESTIMATED CONCENTRATIONS OF SELECTED COMPONENTS OF THE CHROMAFFIN GRANULE LYSATE

Component	Concentration (mol/mol adenine nucleotides)	Concentration (chemical assay)	[Ref.]
Uridine nucleotides	0.08	0.083 (GDP + UDP)	[12]
Guanidine nucleotides	0.13	0.11 (GTP)	[12]
Cytidine nucleotides	<0.02	—	
Ascorbic acid	0.14	0.10	[5]
Dehydroascorbic acid	<0.02	—	
Dopamine	<0.03	0.03	[6]
Lipid olefinic carbon	2.7	—	
NMR-visible protein *			
tryptophan	12 *	—	
glutamine	39 *	≤43	[27]

* Values reported are residues per 77 000 dalton unit of NMR-visible soluble protein. Calculation of these values is described in the text.

resolved C-2 (protonated) resonance of ADP and ATP. The ratio of integrals gives an estimate of the molar ratios of GNP and UNP with respect to total adenine nucleotides. In order to verify that the protonated carbons of the nucleotides are fully relaxed, relaxation studies were carried out. T_1 values of all the protonated adenine nucleotide resonances fall in the range 70—80 ms and, thus, these peaks are fully relaxed with the pulse delay used (850 ms). The accuracy of measured concentration ratios should thus be limited by the accuracy with which integrals of GNP and UNP peaks can be measured and is estimated to be 25%. The values obtained are given in Table I.

Assignments for ascorbic acid, dehydroascorbic acid, and dopamine (all at pH 5.5) are given in Figs. 3—5. The C-4 resonance of ascorbic acid is well resolved at 79.5 ppm and demonstrates clearly the presence of reduced ascorbate in the lysate. Other resonances of ascorbate are obscured by overlap. Dehydroascorbate is not present in observable concentration. The concentration of ascorbic acid has been estimated from the integral of the C-4 (protonated) resonance as described above. The value found is 0.14 mol/mol adenine nucleotides, which compares reasonably well with the assayed value of 0.10 mol/mol adenine nucleotide [5]. Dopamine is an established constituent of chromaffin granules [24], but the concentration (0.03 mol/mol adenine nucleotides) is near the detection limit of our measurements. Even the unobscured protonated carbon peak, C-2, is at the noise level.

Chromogranin is highly soluble in aqueous solution and gives assignable resonances for every amino acid residue except cysteine. The pattern of intensities closely matches the amino acid composition of chromogranin A [13,25—27], with certain exceptions that will be pointed out below. Generally, the resonances fall in the following regions: 1, amide and carboxyl carbons (172—182 ppm); 2, aromatic carbons near 130 ppm; 3, α-carbons from 51—61 ppm; 4, side-chain methylenes (20—41 ppm) and 5, side-chain methyls (15—20 ppm). Tryptophan has not previously been measured in amino acid analyses of chromogranin but is evident in resonances at 112.8 (C-7p), 122.8 (C-6p), 125.3

(C-2p) and 137.2 (C-8p). A comparison of the integrals of these peaks (again using spectra without nuclear Overhauser enhancement) with those of other well-resolved resonances of carbons in amino acids of known abundance provides an estimate of the relative abundance of tryptophan. For this measurement the δ-CH_2 peak of arginine was chosen as standard. It was assumed that 53 arginine residues are present per 77 000 dalton unit of chromogranin A [24, 26]. On this basis, we estimate that 12 ± 3 tryptophan residues are present in the same 77 000 dalton unit. Similarly, the relative number of glutamine and asparagine residues has not previously been measured and can be estimated from the γ-Gln methylene peak at 32.3 ppm. A comparison of integrals gives a value of 39 ± 5 glutamine residues per 77 000 dalton unit. The number of asparagine residues is, therefore, expected to be 30 if the value of free amide groups measured by Smith [27] is used. As seen in Fig. 5, the β-Asn peak coincides with the β-Phe, β-Ile and β-Tyr resonances so that a direct confirmation of this estimate using peak integrals is not possible.

Several resonances are observed in the aromatic-olefinic region between 127 and 132 ppm. A close comparison of the amino acid assignments in this region and the expected intensity pattern from the amino acid composition of chromogranin shows that the protein accounts for only a minor portion of the total intensity in this region. The two upfield peaks (131.4 and 131.7 ppm) can be assigned to protonated carbons on tyrosine (C-2) and phenylalanine (four degenerate peaks at C-2, -3, -4 and -6). The remaining seven peaks have a total intensity that is much greater than that attributable to the assigned protein resonances. These peaks almost certainly arise from olefinic carbons in unsaturated fatty acids. Oleic, linoleic, linolenic and arachidonic acids all have resonances between 127 and 131 ppm [19]. Lipid methylene resonances are also evident, though somewhat less obviously, in the upfield region near 30 ppm. The rather broad peak at 31.0 ppm coincides with the position expected for fatty acid side-chain methylene resonances. Since the peak at 30.1 ppm coincides precisely with the δ-Lys peak, and that at 32.0 with the γ-Gln peak, only the β-Val (35 residues) and β-Met (16 residues) carbons are left to explain the intense peak at 31.0 ppm. The intensity of the β-Val peak may be estimated from the resolved γ-Val peak at 20.0 ppm. The β-Met intensity is only 45% of that of β-Val. Clearly, the intense peak at 31.0 ppm is produced primarily by non-amino acid resonances, and thus may be assigned principally to intensity from fatty acid methylene carbons, which, in ^2H_2O, fall in the range 130—131.1 ppm [28]. It is difficult to make further statements concerning the nature of the soluble lipid. Distinct choline methyl resonances are not observed at 55—56 ppm. Methyl resonances are usually quite narrow and it is unlikely that they would be obscured by other resonances in this region. The concentration of lipid in the aqueous phase appears to be substantial. If all the intensity between 127 ppm and 130.2 ppm is assigned to olefinic lipid carbons, then the concentration estimated from integrals is 2.7 mol olefinic carbon/mol adenine nucleotide.

A few minor peaks have not yet been assigned. These occur principally in the nucleotide base region at 147.2, 146.5 and 145.8 ppm, and in the carbohydrate region at 89.4, 86.4 and 84.1 ppm. We are unable to assign these peaks definitively but suspect that they arise from adenine-containing oliogonucleo-

tides. Two of the resonances in the sugar region are close to those found for C-1′ and C-4′ in 3′-AMP and 3′,5′-adenine dinucleotides [19]. In the base region, large upfield shifts of approx. 2.5 ppm occur in C-4 and C-8 adenine resonances of poly(A) due to base-stacking [29]. These shifts are consistent with the pattern of unexplained resonances in the lysate. Unfortunately, the available reference data are insufficient to permit an unambiguous assignment.

Conclusions

The composition of the chromaffin granule lysate as observed by ^{13}C-NMR corresponds remarkably well with the tabulation of established soluble components listed by Winkler [13]. Table I contains a compilation of concentrations, as estimated by NMR, of selected soluble components including the previously undetected species. Results from classical assays have been included for comparison where available. The spectra confirm the presence of guanidine and uridine nucleotides at levels of approx. 0.13 and 0.08 mol/mol adenine nucleotide, respectively. The concentrations of cytidine nucleotides and nicotinamide nucleotides are below the detection limit of 0.02 mol/mol adenine nucleotide.

Components other than those listed by Winkler may also contribute resolvable intensity to the spectrum. It has been reported that chromogranin A, dopamine-β-hydroxylase and other soluble proteins of the aqueous phase are glycoproteins [36]. The soluble proteins minus dopamine-β-hydroxylase are relatively rich in galactose, *N*-acetylgalactosamine and sialic acid. The hexone and oligosaccharide regions (160—164 and 69—78 ppm) are strongly obscured by sucrose, but a number of resolved peaks of lower intensity are apparent, particularly in the range 70—74 ppm. The majority of these peaks are unassigned, and probably arise from unidentified carbohydrate resonances.

Solubilized lipid is undoubtedly present in the lysate and contributes intensity at 127—131 and 30.5—31.5 ppm. The observed peaks are quite sharp and indicate high segmental mobility and an isotropic environment. It is unlikely that these resonances arise from unsedimented membrane fragments. The possible presence of lipoprotein in the chromaffin granule aqueous phase has been the subject of considerable controversy [13,30—33]. Koenig [31] and Helle [32] have claimed that chromogranin is a lipoprotein. Winkler [13] has pointed out that the measured lipid content of the lysate is extremely variable, however, and has viewed this claim skeptically. Furthermore, he has reported [34] that only 1.4% of the total phospholipid remains in the aqueous phase after centrifugation at $180\,000 \times g$ for 60 min. Recently, Hogue-Angelletti and Sheetz [35] have isolated high molecular weight lipoprotein complexes from the lysate.

The ^{13}C spectra support Helle's original suggestion that lipoprotein is present in the aqueous phase. The NMR-visible lipid fraction contains a high proportion of unsaturated fatty acids, but, surprisingly, a resolved resonance assignable to choline methyls is not apparent. In whole medullary tissue and in intact chromaffin granules the methyl resonance of phosphatidylcholine in membranes is intense and is resolved from the neighboring epinephrine methylene resonance. This suggests that the phosphatidylcholine content of the lipoprotein is small.

Rather unexpectedly, the ^{13}C spectrum contains several unassigned resonances of similar intensity that lie in the general regions of both base and ribose peaks of purine nucleotides. The intensity of these resonances is comparable to that from guanidine and uridine nucleotides, which are present at concentrations near 10 mM. The pattern of chemical shifts suggests that a low molecular weight, adenine-containing oligonucleotide may be present, but the available reference data do not permit an unambiguous identification.

Acknowledgements

Partial financial support of this work was provided by grants from the Michigan Heart Association and from the Rackham School of Graduate Studies of The University of Michigan. We thank Bruker Inc. and Varian Associates for their kind assistance in obtaining spectra.

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