Acta Chemica Scandinavica B 32 (1978) 8–14

Distribution of CAP * and Methylated Nucleotides between the RNase Sensitive and Resistant fractions of mRNA in Messenger Ribonucleoprotein Particles

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When polyribosomal mRNP is exposed to ribonucleases most but not all of the mRNA is converted to acid soluble products. If mRNP is prepared under isotonic conditions there are two types of ribonuclease resistant core fractions, one which contains the poly(A) part of the mRNA and a second which contains mRNA fragments, 30 – 40 nucleotides in length. Like poly(A) these fragments appear to be protein associated in the mRNP complex. Non-poly(A) fragments in mRNP prepared from adenovirus-infected cells harvested in the late phase of infection contained only 3 % of CAP structures and 12 % of internally located methylated nucleotides. This indicates that no CAP structures but one out of the seven internally located methylated nucleotides found in the mRNA are situated in protein associated regions.

Over the past ten years evidence has accumulated suggesting that heterogeneous nuclear RNA as well as messenger RNA of animal cells exist as ribonucleoprotein particles.[1] Polysomal messenger ribonucleoprotein (mRNP) particles prepared under high salt conditions contain two main polypeptides with molecular weights of 78 000 and 50 000.[2–6] Polysomal particles prepared under isotonic conditions contain additional polypeptides.[3,7–9] The polypeptide with a molecular weight of 78 000 is the only polypeptide in mRNP whose binding site on mRNA is reasonably ascertained. It is found in association with the 3′ terminal poly(A).[10–12] In mRNP prepared under high salt conditions all of the mRNA except the poly(A) sequence appears to be available for RNase digestion.[11] In mRNP isolated under isotonic conditions most of the mRNA is also available for digestion.[13] However, in this case there is a fraction of the mRNA, in addition to the poly(A), which is resistant to digestion. This additional fraction in the mRNA consists of several short fragments. Thus there seems to be domains in the mRNA, other than the poly(A) sequence, which are associated with mRNP-proteins.[13] Where these RNase resistant non-poly(A) fragments are located in the mRNA, and which of the mRNA-associated proteins bind to them is not known.

We have used adenovirus-infected cells as a model system in our mRNP studies. Analyses by others [15–17] of [^{3}H]methyl labeled cytoplasmic mRNA from such infected cells have shown that the mRNA contains methylated residues in the form of CAP 1 and CAP 2 structures [14] and at internal locations in the mRNA. In an attempt to look for specific traits in the non-poly(A), RNase resistant sequences in mRNA we have analysed the RNase sensitive and resistant mRNA fractions of mRNP for the presence of CAP structures and internally located methylated nucleotides.

EXPERIMENTAL

Cell fractionation and labeling procedures. HeLa cells were grown in suspension cultures at cell densities of $(2-4)\times10^5$ cells per ml and infected with adenovirus type 2 as described earlier.[18] Infected cells were labeled between 14

* CAP = specialised 5′-terminus in mRNA.[14]

and 16 h after infection with [^{3}H]uridine (25 Ci/mmol) or [^{3}H]adenosine (25 Ci/mmol) at 15 μCi/ml or with [^{32}P]orthophosphate (50 mCi/mmol) at 50 μCi/ml. Cells were labeled with [*methyl*-^{3}H]methionine between 14 and 17 h after infection as follows: Infected cells were grown in spinner medium containing 20 μM methionine, 30 μM adenosine and 7 % serum. At 14 h after infection the cells were centrifuged and washed twice with methionine-free medium and suspended in one-tenth the original volume of medium containing 20 mM sodium formate, 20 μM adenosine, 20 μM guanosine and 50 μCi/ml of [*methyl*-^{3}H]methionine. The cells were lysed with 0.65 % Nonidet P40 in 0.15 NaCl, 10 mM tris-HCl pH 7.8, 1.5 mM $MgCl_2$ and lysates were centrifuged 10 min at 16 000 *g* to remove nuclei and debris. Polysomes were then prepared from the supernatant lysates as described previously.[18] Radiochemicals were purchased from Amersham, England.

Isolation of mRNP. Polysomes were dissociated with 0.03 M EDTA, diluted 5-fold with buffer (final concentrations of 0.2 M NaCl, 50 mM tris-HCl pH 7.8, 10 mM EDTA and 0.5 % Nonidet P40) and fractionated on oligo (dT)-cellulose at 4 °C.[13] Unadsorbed ribosome subunits and tRNA were washed out off the oligo (dT)-cellulose column with a buffer containing 0.2 NaCl, 50 mM tris-HCl pH 7.8 and 10 mM EDTA (buffer A). Adsorbed mRNP was then released from the column with 1 % sarkosyl in 10 mM tris-HCl pH 7.8 (buffer B) at 37 °C. More than 95 % of adsorbed material was recovered with this buffer.

Ribonuclease digestion of polysomal mRNP adsorbed to oligo (dT)-cellulose. Polysomal mRNP adsorbed to oligo (dT)-cellulose was exposed to RNase A (1 μg/ml) and RNase T1 (1 unit/ml) by introducing one column volume of buffer A containing the enzymes. The flow was stopped and the enzymes allowed to act for 60 min, after which the column was again opened and washed with the same buffer until eluted fractions contained near background radioactivity. Less than 5 % of the mRNP-proteins were released from the matrix during this treatment.[18] After several column volumes of buffer A (minus RNases) had passed the column the absorbed mRNP cores were eluted with buffer B. The recovery of mRNP cores was more than 95 %.

Deproteinising of mRNP cores recovered after ribonuclease treatment. For the analysis of [^{3}H]-methyl labeled nucleotides in mRNA it is important to use deproteinised RNA since most of the label from [*methyl*-^{3}H]methionine is introduced into proteins and radioactive protein components easily contaminate in the final analysis. Several of the polypeptides associated with mRNA are tightly bound to the nucleic acid.[19] Evans and Rosenfeld [20] reported that even after SDS-phenol-chloroform extraction small amounts of the 78 K polypeptide is found on mRNA. We have tested several procedures for deproteinising RNase-treated mRNP fractions. The procedure of Holmes and Bonner [21] is useful for deproteinising full length mRNA, but it was difficult to recover RNase digestion products from the extraction medium in a form suitable for further analysis. The hot phenol-SDS procedure [22] gave relatively low recoveries of RNA digestion products and to increase the recoveries we treated samples with proteinase K before the phenol extraction.[23] This, however, led to the appearance of labeled fragments of protein which could not be removed from the RNA fragments by phenol extraction and which interfered severely with the subsequent analysis for methylated nucleotides. Palmitier described a procedure [24] where the nucleic acid-protein mixture first is shaken with phenol at acidic pH and then with phenol/chloroform. Chloroform is thereby prevented from inducing formation of insoluble protein/RNA aggregates which invariably leads to losses of RNA. The following modification of Palmitier's method gave pure RNA and was subsequently used for all analyses. The fractions were incubated for 10 min at 37 °C in 0.1 NaCl, 1 % sarkosyl, 10 mM EDTA and 20 mM tris-HCl pH 7.8. One volume of freshly distilled phenol was added and the mixture was shaken for 5 min at room temperature. Then one volume of chloroform-isoamyl alcohol (99:1) was added and the sample shaken for another 5 min. The water phase was saved. The organic phase and the interphase were extracted with one volume of the starting buffer. The combined water phases were re-extracted with one volume of phenol and one volume of chloroform-isoamyl alcohol added in sequence as described above. The water phase was then shaken twice with chloroform-isoamyl alcohol alone and finally 10 times with 2 volumes of anhydrous ether. The ether of the water phase was removed by a stream of air led over the sample.

Polyacrylamide gel electrophoresis. Samples to be analysed by polyacrylamide gel electrophoresis were brought to pH 5.0 by the addition of 0.1 M NaAc, pH 5.0. Ribosomal RNA (5 μg/ml) was added as carrier and the RNA was precipitated with 2 volumes of -20 °C ethanol. The RNA was pelleted (25 000 *g*; 60 min) and dissolved in 0.1 × SSC, 0.5 % sarkosyl. Formamide was added to a final concentration of 80 % and the samples were incubated at 37 °C for 5 min. After chilling and dilution (to 50 % formamide) of the samples with 0.1 × SSC, 0.5 % sarkosyl they were applied to polyacrylamide gels. The electrophoresis was performed either in 12 % polyacrylamide (for fragments) or 2.2 % polyacrylamide-0.8 % agarose gels (for full length RNA).[18] The gels were cut in 2 mm slices, incubated with 0.1 M NaOH for 60 min and then suspended in toluene-methanol based scintillation liquid for determination of their radioactivity.

DEAE-cellulose chromatography. Fractionation of [^{3}H]methyl labeled nucleotides was

performed on DEAE-cellulose as follows: DEAE-cellulose (Whatman DE 52) was pretreated with 7 M urea, 0.3 NaCl in 20 mM tris-HCl pH 7.6, and then washed free of salts. Deproteinised mRNA fractions were incubated in 0.3 M KOH for 18–24 h at 37 °C. After neutralisation with perchloric acid and chilling, insoluble $KClO_4$ was removed by centrifugation. The samples were diluted with water and made 7 M in urea and 20 mM in tris-HCl at a final pH of 7.6, giving samples of 750 ml and with a conductivity of 1.5–2 mS. They were then applied to 1 × 20 cm DEAE-cellulose columns, which were developed with 200 ml gradients: 0–0.3 M NaCl in 7 M urea, 20 mM tris-HCl pH 7.6. Fractions of 2 ml were collected and analysed for absorbance at 260 nm and for radioactivity in Instagel (Packard Instrument Co.). The presence of labeled mononucleotides in the flowthrough after application of the samples on the columns and the unusually broad peak of mononucleotides which extends even into the dinucleotide position in Fig. 3 can probably be attributed to the high salt content of the samples. Due to the presence of methylated ribose in rRNA, alkali digestion of [^{3}H]methyl labeled rRNA would give rise to labeled dinucleotides. However, the material extending into the dinucleotide position in Fig. 3 does not contain any dinucleotides, as shown by the following two criteria: (a) The mononucleotide marker also extends into the dinucleotide position under the conditions in Fig. 3 and the degree of this extension parallels that of the labeled mononucleotides; (b) if samples corresponding to those shown in Fig. 3B and 3C are precipitated with ethanol about 50 % of the material is recovered in the precipitate. This precipitation procedure will not distinguish between material from mRNA and rRNA. However, subsequent alkali digestion and DEAE-cellulose chromatography of the desalted material revealed no radioactivity in the flowthrough or in the dinucleotide position but sharp peaks of radioactivity corresponding to mononucleotides and CAP's.

RESULTS AND DISCUSSION

Occurrence of methylated nucleotides in non-poly(A), RNase resistant fragments of mRNP. During the late phase of adenovirus-infection synthesis of host mRNA and ribosomal RNA is strongly suppressed.[25] Eighty to 90 per cent of the newly synthesised polysomal mRNA is virus coded,[18] and the major part of the protein synthesising capacity of the cell is devoted to the production of viral polypeptides. Ribosomal RNA and polysomal mRNA from such adenovirus-infected cells labeled with [*methyl*-^{3}H]-methionine are displayed in Fig. 1A and B,

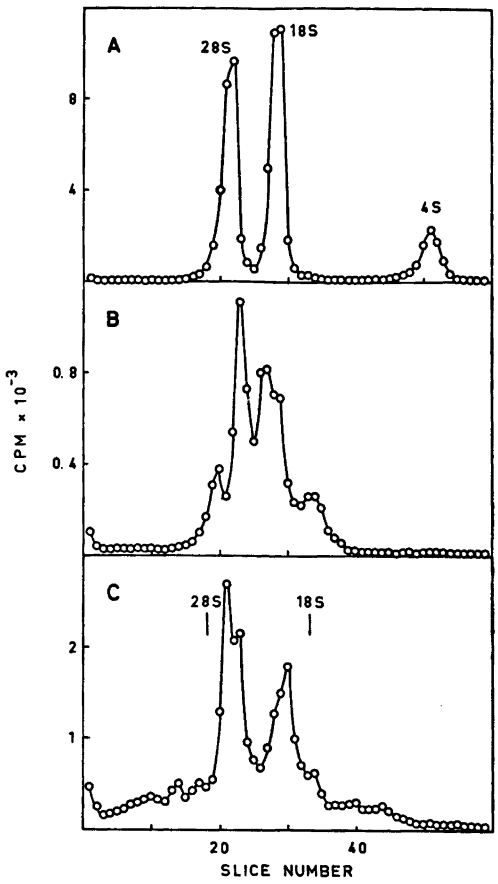

Fig. 1. Size distribution of mRNA derived from polysomal mRNP. Polysomes were deproteinised and fractionated on oligo(dT)-cellulose.[27] Flowthrough and adsorbed material were recovered from the column, precipitated and subjected to electrophoresis in polyacrylamide-agarose gels as described in the Experimental section. Results displayed in panel A and B represent flowthrough and adsorbed fractions respectively, from [*methyl*-^{3}H]methionine labeled cells. Panel C shows the analysis of mRNA from oligo(dT)-selected mRNP from [^{3}H]uridine labeled cells.

respectively, and compared with [^{3}H]uridine labeled mRNA derived from oligo(dT)-cellulose selected mRNP (Fig. 1C). The [^{3}H]methyl labeled mRNA gave rise to a pattern of peaks which closely resembled that of [^{3}H]uridine labeled mRNA. There are no obvious signs of cross contamination between the rRNA and mRNA fractions (Fig. 1A and B). Since there is no [^{3}H]methyl label at the position of 5 S

rRNA it seems safe to assume that no label has been introduced into the RNA *via* the carbons in the purine rings.[26]

Polysomal mRNP labeled with [*methyl*-^{3}H]-methionine was then analysed for the presence of methylated nucleotides in poly(A) and in the non-poly(A), RNase resistant mRNA fragments as well as in the RNase sensitive mRNA fraction. For this purpose polysomes were dissociated with EDTA and the sample passed through an oligo(dT)-cellulose column. The adsorbed mRNP was then exposed to ribonucleases A and T1 and the RNase resistant structures eluted from the resin with buffer B as described in the Experimental section. As illustrated further below (Fig. 3 and Tables 1 and 2), most of the [^{3}H]methyl labeled nucleotides were recovered in the RNase sensitive mRNA fraction. However, some 8 % of the methylated nucleotides were recovered in RNase resistant fragments. These [^{3}H]methyl labeled mRNA fragments were deproteinised, concentrated by ethanol precipitation, treated with formamide and finally subjected to polyacrylamide gel electrophoresis as described in the Experimental section. The results of this analysis are shown in Fig. 2 together with results obtained in parallel analyses of [^{3}H]-adenosine and [^{3}H]uridine labeled material. The [^{3}H]methyl labeled fragments gave rise to a peak (Fig. 2C), which migrated with approximately the same characteristics as that obtained with [^{3}H]uridine labeled RNA fragments (Fig. 2B). In addition to the poly(A) a similar peak is also seen in the analysis of the [^{3}H]adenosine labeled RNA (Fig. 2A). By comparison of the migration of the 5 S rRNA and tRNA markers, the length of the non-poly(A) fragments was estimated as 30 – 40 nucleotides. The absence of [^{3}H]methyl labeled material in the position expected for poly(A) (Fig. 2C) corroborates an earlier report that there are no methylated residues in poly(A) of adenovirus mRNA.[15]

Invariably we find small amounts (2 %) of the [^{3}H]uridine labeled RNA fragments comigrating with poly(A) (Fig. 2B). These uridine-containing RNA fragments bind to poly(U)-Sepharose and after elution and treatment with formamide they again comigrate with poly(A) in polyacrylamide gels (data not shown). No label is found at the poly(A) position

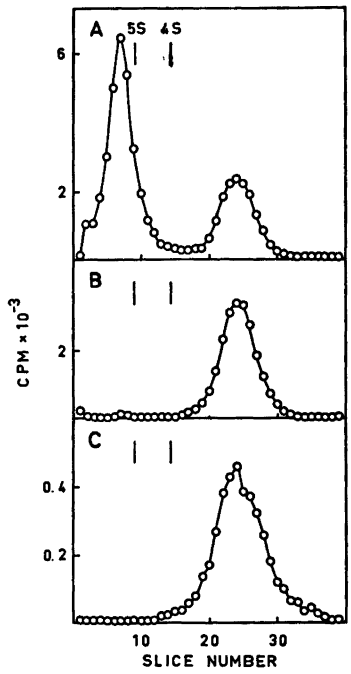

Fig. 2. Size distribution of mRNA fragments obtained after RNase digestion of mRNP. Polysomal mRNP from adenovirus-infected cells labeled in various ways was adsorbed to oligo(dT)-cellulose and digested with RNases A and T1 as described in the Experimental section. The resulting mRNP cores were recovered from oligo(dT)-cellulose, deproteinised and analysed as detailed in the Experimental section. Panel A shows [^{3}H]adenosine labeled fragments and B and C material labeled with [^{3}H]uridine and [^{3}H]methyl, respectively. The positions of 5 S rRNA and tRNA run on parallel gels are indicated. In this particular experiment the [^{3}H]uridine and [^{3}H]adenosine labeled mRNPs were prewashed with 10 mM tris-HCl pH 7.8 before RNase digestion during buffer A-conditions. This low-salt wash removed 10 % of intact mRNP-particles, but did not effect the final results of RNase digestion.

when deproteinised [^{3}H]uridine labeled mRNA is examined after RNase digestion, suggesting that the association between poly(A) and the poly(A)-binding proteins also confers RNase resistance to an additional short sequence in juxtaposition to poly(A).

Distribution of CAP and methylated mononucleotides between RNase sensitive and resistant fractions of mRNA in mRNP. The observation that the 30 – 40 nucleotide long fragments

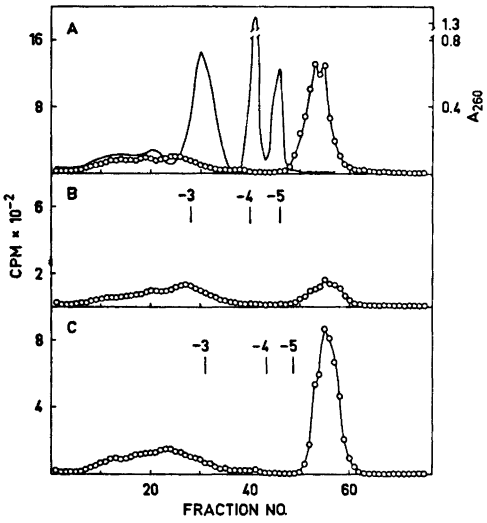

Fig. 3. Distribution of CAPs and methylated nucleotides between the RNase sensitive and resistant fractions of mRNA in mRNP. Polysomal mRNP from cells labeled with [*methyl*-^{3}H]methionine was adsorbed to oligo(dT)-cellulose and digested with RNases A and T1. Material removed from the column with RNases, resistant mRNA-protein cores eluted with buffer B and undigested mRNP were deproteinised as described in the Experimental section. The samples were then treated with alkali and made 7 M in urea and 20 mM tris-HCl pH 7.6. Markers for mono-, di-, tri- and tetraphosphate were added and the samples were then fractionated on DEAE-cellulose columns. Panel A shows the methylated products in the RNase sensitive fraction of mRNP, panel B those of the RNase resistant mRNP cores and panel C shows the analysis of the undigested mRNP not treated with RNases. —, A_{260} nm; O, radioactivity.

contain methylated nucleotides called for a more detailed analysis with respect to the presence of CAP structures and methylated mononucleotides. For this polysomal mRNP was adsorbed to oligo(dT)-cellulose and digested with RNase A and T1. After removal of the nuclease sensitive material the resistant mRNP cores were recovered from the oligo(dT)-cellulose with buffer B. The RNase sensitive and resistant material was deproteinised, hydrolysed with alkali and subjected to chromatography on DEAE-cellulose columns as described in the Experimental section. The results of such an analysis are shown in Fig. 3 and Table 1. A small but significant amount (12 %) of the methylated mononucleotides was recovered in the RNase resistant fraction although the major part was removed from mRNP by the enzymes. Of the [^{3}H]methyl label in CAP structures only 3 % were found in the RNase resistant fraction. Thus the proteins of mRNP apparently are not in close association with the CAP structure, and the major part of methylated mononucleotides of mRNA also seem to lack association with the mRNP-proteins. Assuming that the [^{3}H]methyl groups are incorporated in CAP structures and mononucleotides with the same efficiency and using 3.5 as the mean number of methyl groups that are incorporated in the CAP structures [15] the figures in Table 1 correspond to seven non-CAP associated methylated nucleotides per CAP.

In [^{32}P] labeling experiments (Table 2) 17 % of the mRNA in mRNP was found to be RNase resistant. If the average sized adenovirus mRNA is taken to be 3000 nucleotides long [18] then the [^{32}P] data suggest that the total RNase resistant fragments of mRNA in mRNP contain about 500 nucleotides. The poly(A) of newly synthesised adenovirus mRNA has been estimated to be close to 200 nucleotides thus leaving 300 nucleotides for the additional, presumably protein associated, sequences. Assuming an average base composition of the fragments and the mRNA, the corresponding values for the non-poly(A), RNase resistant material as calculated from the [^{3}H]uridine and [^{3}H]adenosine incorporation data (Table 2) are 250 and 360 nucleotides, respectively. The length of the non-poly(A), RNase resistant fragments is 30 – 40 nucleotides. Thus there appears to be 8 – 10 fragments produced per average mRNA. On average one of these would be in a region of the mRNA that contains a methylated mononucleotide.

Since fragments treated with 80 % formamide (as in Fig. 2) migrate significantly faster than untreated fragments [13] it is suspected that the RNase resistant fragments are derived from regions of the mRNA which have a high secondary structure.

It was reported recently that [^{3}H]methyl labeled cytoplasmic mRNA obtained from cells harvested late after adenovirus-infection has 34 – 46 % of the label in CAP structures, and

Table 1. Distribution of [^{3}H]methyl labeled CAPs and mononucleotides in polysomal mRNP and in the fractions derived after RNase digestion of mRNP. The radioactivity in CAPs and mononucleotides was determined from analyses illustrated in Fig. 3.

Sample [a]	Radioactivity/counts min^{-1}			Radioactivity in CAPs as % of total radioactivity in sample
	Mono-nucleo-tides [b]	CAPs	Total in sample	
Polysomal mRNP (Panel C in Fig. 3)	83 964	43 512	127 367	34.1
Ribonuclease digested polysomal mRNP				
I Fraction eluted with ribonuclease (Panel A in Fig. 3)	72 546	39 976	112 522	
II RNase resistant core fraction (Panel B in Fig. 3)	9 738	1 325	11 093	
Total CPM (I+II)	82 314	41 301	123 615	33.4

[a] Calculations of recovered radioactivity are corrected for the same amount of polysomes used in the experiments shown in Fig. 3A, B and C. [b] These figures include the radioactivity found in the flowthrough after application of the samples on DEAE-cellulose (see Experimental section).

Table 2. RNase resistant sequences in mRNA-protein complexes from adenovirus-infected cells. The conditions for labelling with the various precursors are given in the Experimental section.

Sample	Messenger ribonucleoprotein particles adsorbed to oligo (dT)-cellulose [a]. Total radioactivity in RNA/counts min^{-1}		
	Before RNase	After RNase	Resistance %
mRNP-[*methyl*-^{3}H]methionine	80.492	5.850	7
	123.615	11.093	9
[^{32}P]mRNP	2131.900	391.500	18
	1684.200	279.000	17
	7132.200	1196.100	17
mRNP-[^{3}H]uridine [b]			9
mRN*A*-[^{3}H]uridine [b]			0.5
mRNP-[^{3}H]adenosine [b]			28
mRN*A*-[^{3}H]adenosine [b]			15

[a] Polysomes were dissociated with EDTA, the mRNP adsorbed to oligo(dT)-cellulose and digested with RNase A and T1 as described in the Experimental section. [b] The experiments behind these results were reported elsewhere.[3] The values are inserted for comparison of suseptibility of mRNP and deproteinised mRNA to the ribonucleases. The 15 % resistance of [^{3}H] adenosine labeled mRNA all resides in the poly(A) sequence.[18]

that the remaining label is in methyl groups in internally located m^{6}A and m^{5}C.[15] The distribution of methyl groups in CAP structures and mononucleotides in polysomal mRNA prepared during the late phase of adenovirus-infection (Table 1) is in agreement with that reported for cytoplasmic mRNA.

Acknowledgements. The author is indebted to Dr. U. Lindberg for his interest and valuable discussions during the course of this investigation and to Miss S. Eriksson and Miss I. Svensson for their skilful technical assistance. This investigation was supported by grants to U. Lindberg from the Swedish Cancer Society, the Knut and Alice Wallenberg's Foundation, Stockholm, Sweden and Uppsala University.

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Received June 27, 1977.