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RIBONUCLEIC ACID METABOLISM DURING THE DEVELOPMENT AND RIPENING OF TOMATO FRUITS

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato *r*RNA; polyadenylic acid-containing RNA; fruit ripening; protein synthesis.

Abstract—The accumulation of RNA in the outer locule tissue of tomato fruits was measured during development and ripening. Labelling studies suggest two peaks of synthesis, the first during early development and the second just before the onset of ripening (colour change). During the second period of increased RNA labelling the amount of total RNA per fruit either remains constant or starts to decline. Synthesis of *r*RNA and soluble RNA occurred at all stages. Polydisperse RNA containing polyadenylic acid was isolated and shown to direct the synthesis of protein *in vitro*. No significant changes in the amount of polyadenylic acid, relative to total RNA were detectable during the ripening period.

INTRODUCTION

There is considerable evidence that changes in enzyme synthesis may be involved in fruit ripening [1]. Hulme *et al.* [2] suggested that in climacteric fruits increased synthesis of malic enzyme and pyruvic carboxylase is associated with ripening and Hobson [3] showed that during the early stages in tomato ripening there is a rapid rise in polygalacturonase activity. In addition, cycloheximide, an inhibitor of protein synthesis on 80S ribosomes, prevents ripening in pears [4], citrus fruits [5] and bananas [6]. More direct evidence for the involvement of protein synthesis is provided by the observation that enhanced incorporation of radioactive amino acids into proteins occurs during the early climacteric in avocado pear slices [7] and whole pears [4], bananas [8], apples [9] and tomatoes [10]. Richmond and Biale [7] interpreted their results as evidence for the "introduction of a set of enzymes which catalyse the climacteric process and the final breakdown of the cell". A similar stimulation of incorporation of radioactive precursors into RNA just prior to the climacteric peak in apples [9, 11], pears [12, 13] and tomatoes [10] supports the suggestion that RNA synthesis is also required for the synthesis of new enzymes involved in ripening. However, in most cases the main type of RNA synthesized appears to be *r*RNA and there is no clear evidence relating to changes in *m*RNA.

This question is of considerable importance because the synthesis of new enzymes could be regulated simply by the translational control of pre-existing *m*RNA. Changes in the levels of different *t*RNAs during ripening support the idea of translational control [14]. Alternatively, the synthesis of new *m*RNA just before fruit ripening could also be responsible for new enzyme synthesis. The demonstration that at least some plant *m*RNAs can be purified by virtue of the presence of poly(A) at the 3′ end [15–19] and the fact that they can be translated *in vitro* and the protein products identified [16, 17, 20] raises the possibility that the above theories relating to changes in RNA and protein synthesis during fruit ripening can be directly tested. As a first step we have studied the metabolism of *r*RNA, soluble RNA and poly(A)-containing RNA during the development and ripening of tomato fruits and examined the activity of poly(A)-containing RNA in directing protein synthesis *in vitro*.

RESULTS AND DISCUSSION

RNA accumulation in tomato fruits

The pattern of accumulation of total nucleic acid during the development and ripening of two varieties of tomato fruit is shown in Fig. 1. Polyacrylamide gel electrophoresis of nucleic acid extracted from fruits at different developmental stages showed that DNA accounted for only 5–10% of the total (Fig. 2). Davies and Cocking have previously reported [21] that there is very little cell division or DNA synthesis during tomato fruit development and the results in Fig. 1 are therefore mainly due to a large increase in the amount of RNA per cell. Net accumulation of RNA ceased several weeks before the onset of ripening, measured by the synthesis of lycopene. In the slow ripening variety Minipopella the RNA content remained stable for several weeks and then declined sharply at the onset of lycopene synthesis. However a decrease in RNA content is not necessarily directly related to ripening because in the quick-ripening Amberley Cross variety a substantial reduction in RNA was detected prior to lycopene synthesis (Fig. 1a).

Abbreviations: Poly(A), polyadenylic acid; Oligo (dT)-cellulose, cellulose with oligodeoxythimidylic acid attached.

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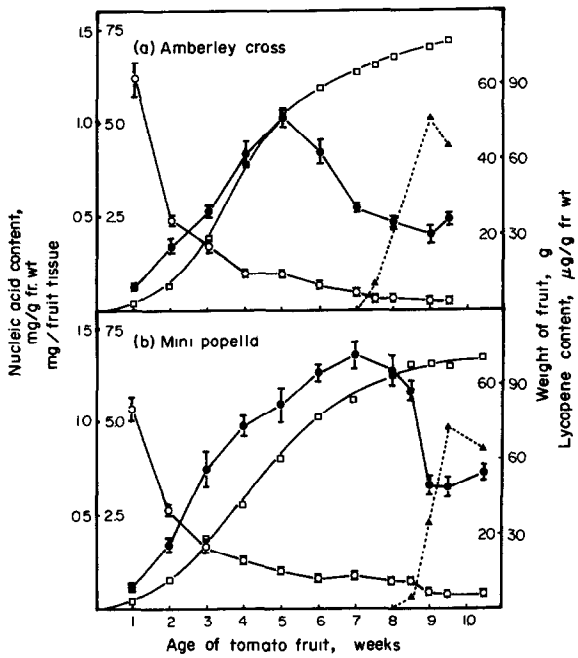

Fig. 1. Changes in fresh weight, RNA and lycopene content during the development and ripening of tomato fruits. Results are shown for a quick-ripening (Amberley Cross, 1a) and a slow-ripening (Minipopella, 1b) variety. □, fresh weight of fruit (g); ●, mg nucleic acid per fruit tissue; ○, mg nucleic acid per g fruit tissue; ▲, μg lycopene per g fruit tissue; Vertical bars indicate standard errors.

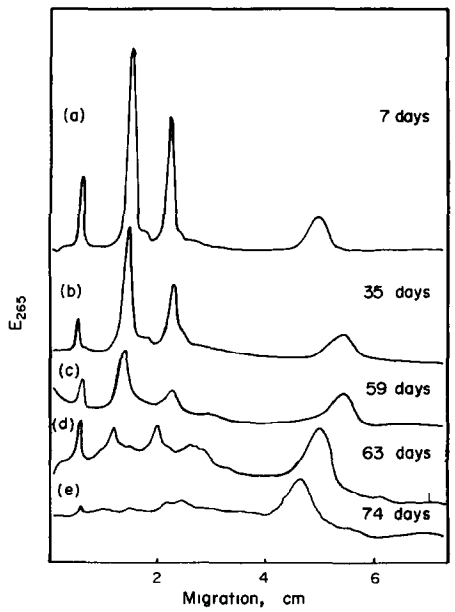

Fig. 2. Polyacrylamide gel electrophoresis of total nucleic acid samples from fruits of different ages. Electrophoresis was in 2.4% gels for 2 hr. All extracts were from the Minipopella variety although similar gel profiles were obtained with Amberley Cross. Curves c and d cover the period immediately prior to and during ripening. In curve a, the peak at 0.6 cm is DNA, the 26S and 18S *r*RNAs are at 1.6 and 2.2 cm and the 23S and 16S plastid *r*RNAs appear as shoulders to the right side of each main peak. The broad peak at 4.9 cm is *t*RNA and 5S RNA.

Polyacrylamide gel electrophoresis showed (Fig. 2) that 66–78% of the RNA, calculated by peak area measurements of the gel scans, was *r*RNA from 80S cytoplasmic ribosomes. Plastid *r*RNA, which migrates slightly faster than cytoplasmic *r*RNA during gel electrophoresis (22) accounted for 3.5–7% and soluble RNA for 12–22% of the total. This compares with estimates of the amount of poly(A)—containing *m*RNA (see below) of *ca* 2.5%. RNA extracted from ripening fruits showed evidence of degradation, judged by the disappearance of *r*RNA and the increase in the amount of soluble RNA (Fig. 2e). For all RNA extractions the tissue was homogenized directly with a phenol-detergent mixture designed to inhibit ribonucleases and the degradation detected by gel electrophoresis is due probably to the action of ribonucleases *in vivo* during tissue breakdown which accompanies ripening. The increase in the relative size of the soluble RNA peak in older fruits is probably brought about by the production of low MW fragments of ribosomal RNA which co-migrate with *t*RNA. Direct evidence for the breakdown of *r*RNA *in vivo* to produce such fragments comes from DNA–RNA hybridization studies with *Matthiola incana* nucleic acids (Grierson and Hemleben, unpublished).

Incorporation of uridine-[^3H] into RNA

Using segments of the slow-ripening Minipopella variety, labelled for 24 hr periods with uridine-[5-^{3}H], evidence for two major periods of incorporation into RNA was obtained (Fig. 3). The first is associated with the rapid increase in fruit size and fr. wt which is accompanied by a marked accumulation of RNA. This is followed by an apparent reduction in the rate of RNA synthesis, which coincides with the period when net accumulation ceases. A second burst of incorporation is initiated 1–2 weeks before the onset of lycopene synthesis, reaches a peak and then declines rapidly. This second peak probably corresponds to the climacteric rise in RNA labelling previously noted in tomatoes [10]. For these labelling studies, large segments of tissue were used. In this way it was hoped to minimize any wound effect due to excessive slicing of the tissue. Labelling of intact fruits by direct injection of radiochemical with a syringe was not effective.

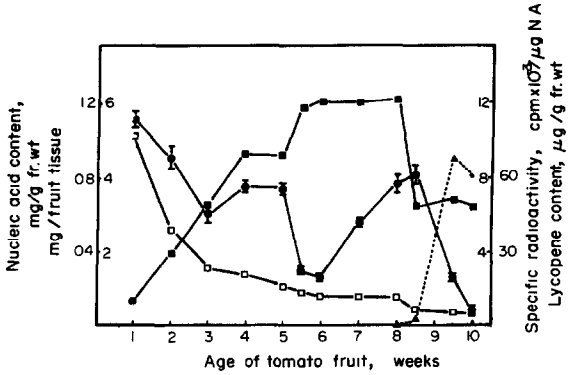

Fig. 3. Rate of labelling of RNA in segment of tomato fruit at different developmental stages. The Minipopella variety was used. ■, mg nucleic acid per fruit tissue; □, mg nucleic acid per g fruit tissue; ●, specific radioactivity (cpm per μg nucleic acid) ▲, μg lycopene per g fruit tissue.

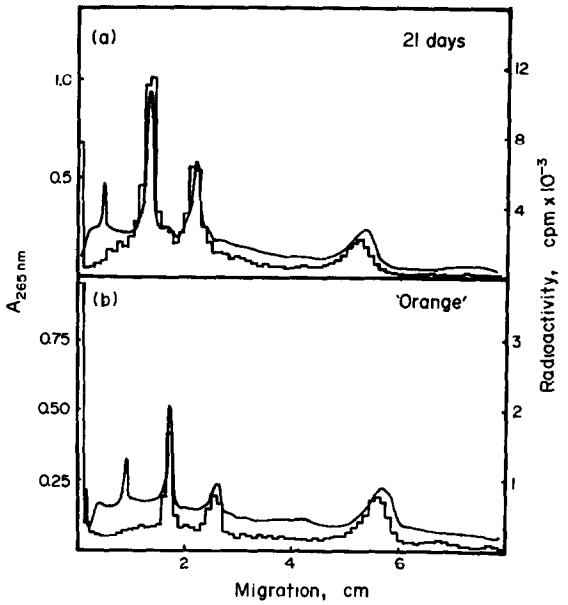

Fig. 4. Incorporation of uridine-[5-^{3}H] into different RNA fractions in unripe and ripening fruit. Unripe (4a) or ripening (4b) tissue segments were labelled with uridine-[5-^{3}H] for 24 hr. Electrophoresis was in 2.4% acrylamide gels for 2 hr. Smooth curve, A_{265} nm; histogram, ^{3}H cpm per mm slice.

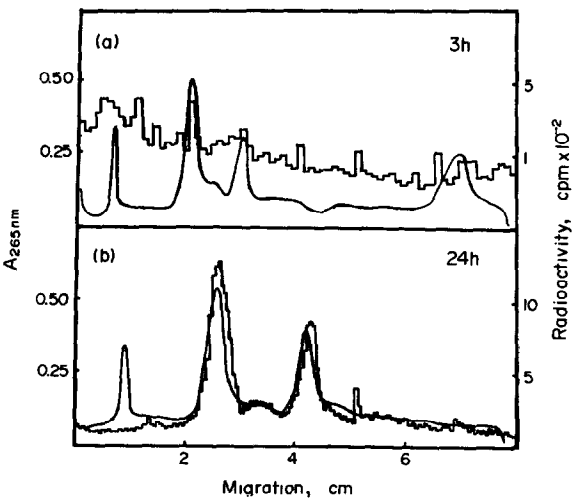

Fig. 5. Comparison of RNAs labelled for different lengths of time. Tissue segments of 6-week-old fruit of the Minipopella variety were labelled for 3 hr (a) or 24 hr (b). Electrophoresis was in a 2.4% gel for 2 hr (a) and 2.6% gel for 4 hr (b). In (b) the soluble RNA was run off the bottom of the gel. Smooth curve, A_{265} nm; histogram, ^{3}H cpm per mm gel slice (a) and 1/2 mm gel slice (b).

Fractionation by gel electrophoresis of labelled RNA samples obtained at weekly intervals during the period shown in Fig. 3 revealed that *r*RNA, soluble RNA and polydisperse RNA were all synthesized at each stage, even during the period of lycopene synthesis when the labelling of RNA is rapidly declining. Representative gel profiles for 24 hr labelling period are shown in Fig. 4. With shorter labelling periods more polydisperse RNA was evident. For example at 6 weeks, when the labelling of RNA is at a minimum (Fig. 3), a 3 hr label revealed largely polydisperse RNA (Fig. 5a), and small peaks (in Fig. 5a at 0.5 and 1 cm) which may represent ribosomal RNA precursor [29]. A detailed examination of similar pulse-labelled polydisperse RNA from sycamore cell nuclei suggests that this type of labelling pattern is due mainly to incomplete chains of *r*RNA precursor, together with a small amount of poly(A)-containing RNA [23]. In tomato fruits, labelling for a longer period of time confirmed that large amounts of *r*RNA are synthesized (Fig. 5b) and that the polydisperse pattern in Fig. 5a obtained after short labelling periods can not be interpreted to indicate the predominant synthesis of *m*RNA.

With the exception of the first week of growth, very little labelling of plastid *r*RNA was detected at any stage of fruit development. This is surprising in view of the suggestion by Davies and Cocking [24] that plastids may be a major site of protein synthesis in tomato fruits. The explanation for the lack of labelling may be that chloroplast *r*RNA is extremely stable and turns over much more slowly than cytoplasmic *r*RNA [25].

The question arises as to the significance of the second burst of RNA labelling shown in Fig. 3. In several climacteric fruits this has been interpreted as evidence for the synthesis of new types of RNA necessary for ripening [9–12]. Our results suggest that the main type of RNA produced is *r*RNA. It is difficult to reconcile this with the hypothetical requirement for new informational RNA (*m*RNA) unless it is assumed that the synthesis of *r*RNA and *m* RNA are obligatorily coupled.

A further difficulty is that although the incorporation experiments suggest a period of increased synthesis, sustained over a period of 1–2 weeks, there is no net increase in RNA content (Fig. 3). The results in Table 1 show that during the period of colour change, charac-

Table 1. Uptake and incorporation of uridine-[^{3}H] during ripening of tomato fruits

	Age of tomato fruits				
	54 days	56 days	57 days	58 days	63 days
Fresh weight of fruit (g)	73.7	73.1	83.7	75.7	77.4
Lycopene content (μg/g fr. wt)	0	1.5	12.5	28.9	70.3
μg RNA/g fr. wt	115	99	73	63	56
mg RNA/fruit	5.19	4.63	3.72	2.95	2.72
Uptake of Uridine-[^{3}H] (10^{-3} × cpm/g tissue)	1490 ±27.7	1690 ±135.0	1300 ±14.3	1500 ±24.3	1550 ±189
Incorporation of Uridine-[^{3}H] (cpm/μg RNA)	4890 ±339	4760 ±385	4000 ±453	4080 ±396	2090 ±109

terized by an initial high level of RNA labelling, followed by a sharp drop, changes in the incorporation of uridine-[^{3}H] into RNA cannot be explained by differences in uptake of radiochemical due to permeability changes. Therefore, if there is a genuine increase in RNA synthesis during the climacteric, it must be assumed that this is balanced exactly by a corresponding increase in the rate of RNA degradation so that no net accumulation occurs. Measurements of the activity of ribonucleases before and during the climacteric are in progress in order to test this hypothesis. An alternative explanation is that the increased labelling of RNA merely reflects an increase in specific radioactivity of the intracellular pool of UTP, the substrate for RNA synthesis. This could occur if during the climacteric there is an increased phosphorylation of exogenously supplied uridine-[^{3}H].

Poly(A)-containing mRNA from tomato fruits

Chromatography of RNA labelled for 24 hr with uridine-[^{3}H] on oligo (dT)-cellluose produced a poly(A)-containing RNA fraction amounting to *ca* 2.5% of the total labelled RNA. The mobility of this RNA during electrophoresis (Fig. 6) does not provide a reliable

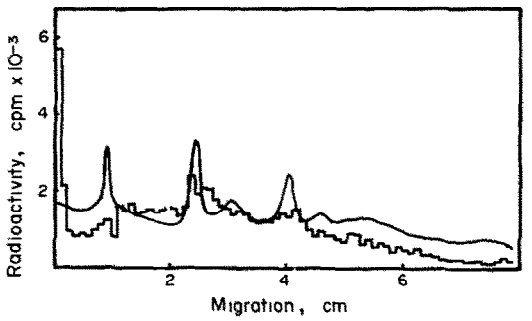

Fig. 6. Gel electrophoresis of poly(A)-containing RNA. Segments from the outer locule tissue of 1-week-old fruits of the Minipopella variety were labelled for 24 hr with uridine-[5-^{3}H]. Poly(A)-containing RNA was purified from the total nucleic acid, mixed with unlabelled carrier nucleic acid and fractionated in a 2.4% gel for 3.5 hr. Smooth curve, A_{265} nm; histogram, ^{3}H cpm mm gel slice.

indication of its MW due to the anomalous behaviour of poly(A)-containing RNA [26]. However, Fig. 6 does show it is polydisperse in size and contains only trace amounts of rRNA. There is substantial evidence that some, but not all, plant mRNAs contains poly(A) (16–19). Poly(A)-containing RNA from tomatoes stimulates the incorporation of radioactive amino acids into protein *in vitro*, confirming that this fraction represents at least part of the mRNA from tomato (Table 2). The protein products synthesized in the wheat germ system *in vitro* in response to tomato fruit mRNA were analysed in 15% acrylamide gels containing urea and SDS. Between 10 and 30 discrete protein bands were detected by autoradiography, ranging in MW from 80000 to 10000 daltons (Rattanapanone, Speirs, Grierson and Stein, unpublished). Poly(A)-containing RNA samples from tomato fruits at different stages of development, and from other plants, show variation in the efficiency with which they stimulate protein synthesis *in vitro*. Preliminary evidence indicates that this is due in some cases to uncharacterized inhibitory substances in the RNA preparations (Speirs and Giles, unpublished). This may explain the variation in efficiency of the two mRNA samples shown in Table 2.

In view of the relationship between mRNA and poly(A)-containing RNA the amount of poly(A) was measured during development and ripening, using a nucleic acid hybridization assay with polyuridylic acid-[^{3}H] [27]. The results are shown in Fig. 7. The gradual decline in

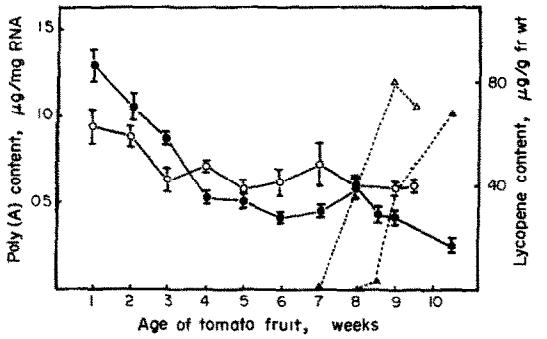

Fig. 7. Change in the amount of poly(A) during fruit development and ripening. ●—●, µg poly(A) per mg RNA (Minipopella); ○—○, µg poly(A) per mg RNA (Amberley Cross); ▲---▲, µg lycopene per g fr. wt (Minipopella); △---△, µg lycopene per g fr. wt (Amberley Cross). Vertical bars represent standard errors.

poly(A) content in older fruits suggests either that there is a shortening of poly(A) segments with age, or alternatively that younger fruits have a higher mRNA to total RNA ratio. The results indicate that there is no large increase in accumulation of poly(A) relative to total RNA during fruit ripening. The slight rise, detected in both varieties just prior to ripening, is not statistically significant (Fig. 7). This suggests that the synthesis of poly(A)-containing mRNA is co-ordinately linked to total RNA production and that the amount of mRNA is relatively constant during the period leading up to the initial events of ripening, when the total RNA content remains constant.

Table 2. *In vitro* protein synthesis activity of tomato fruit Poly(A)-containing RNA

	Control Sample 1*		Sample 2†		
µg of Poly(A) containing RNA per 25 µl assay mixture	—	3.4	1.0	2.5	5.1
Incorporation of radioactive methionine-[^{35}S] (cpm/2µl aliquot)	621	3030	3550	5180	7420

* Small green fruits, age about 2 weeks. † Immature green fruits, age about 4 weeks.

Our measurements take no account of that fraction of *m*RNA, perhaps 50% [18], which lacks poly(A). Furthermore the *m*RNA population in tomato fruits may code for in excess of 100 enzymes. Therefore the synthesis of a relatively small number of new *m*RNAs in small amounts would probably not be detected by the methods employed here. We are currently examining the products of *in vitro* protein synthesis stimulated by poly(A)-containing RNA from fruits at different stages to test whether there are specific changes in *m*RNA content related to ripening.

EXPERIMENTAL

Materials. Tomato plants (*Lycopersicum esculentum* Mill.), Amberley Cross (quick ripening) and Minipopella (slow ripening) varieties were grown in soil in a heated greenhouse at temperatures between 15–25°. Fruits were allowed to set naturally and the age was calculated from the day of pollination, which was taken as the day after closing of the flower [21]. Fruits of a given age were detached from the plant before use. In a single series of experiments comparing fruits at different stages of development the age is given in days or weeks. Where different expts are compared fruits often ripened at different ages. The age in days then becomes meaningless and for comparisons the terms green (unripe) orange (ripening) and ripe are used. Only the outer locule tissue of the fruits was used for analysis.

Extraction and purification of nucleic acids. Tomato fruits were harvested on the day of the experiment and cleaned with tissue paper. The size and wt of the fruit was recorded. Fruits were cut in half and the seeds and gel were removed from the locule. The liquid on the internal surface of the outer locule tissue was carefully dried with tissue paper, taking care not to damage the internal surface. The outer locule tissue was then reweighed and 3–4 g portions from individual fruits were used for analysis. Nucleic acids were extracted and purified from the outer locule tissue by the procedure described in ref. [27] for measurement of poly(A) content. Poly(A)-containing RNA was purified by oligo(dT)-cellulose chromatography [27]. The RNA samples used to obtain the result in Fig. 6 were freed of carbohydrate by centrifugation through LiCl [28]. Total nucleic acid was estimated by measuring the A_{260} nm. A 40 μg/ml soln was assumed to have an $A_{260} = 1$ using a 1 cm light path.

Labelling of RNA. Duplicate segments of outer locule tissue weighing 3–4 g were placed skin downwards on moist filter paper in a Petri dish and 50 μl uridine-[5-^{3}H] (1 μCi/ml; 25 Ci/m mol) was distributed on the inner surface. The incubation period was 3 or 24 hr in a high humidity desiccator at room temp. Uptake of radioactive uridine was measured at the end of the incubation by washing tissue for 5 min in 0.5 mM unlabelled uridine. The washing was repeated twice more with fresh unlabelled uridine soln. Tissue was then homogenized in the detergent mixture used for nucleic acid extraction [27], centrifuged and an aliquot of the supernatant counted in a liquid scintillation counter. Incorporation of radioactivity was measured by TCA (5% final concn) precipitation of aliquots of RNA extracted and purified as indicated above. Ppts were trapped on nitrocellulose filters which were dried and counted in a scintillation counter.

Separation and fractionation of RNA. Polyacrylamide gel electrophoresis was carried out as described in ref. [29] and radioactivity was measured according to ref. [30]. Poly(A)-containing RNA was separated by oligo (dT) cellulose chromatography by the method of ref. [27]. The poly(A) moiety was measured by hybridization with polyuridylic acid-[^{3}H] (27).

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