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In Vitro Iodination of Plant Ribonucleic Acids[1, 2]

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ABSTRACT

The optimum conditions for *in vitro* iodination of RNAs have been established which yield specific radioactivities ranging from 10×10^4 to 10×10^6 cpm/μg. A nomogram has been constructed by correlating specific radioactivities of RNA with concentration of KI, RNA, and ^{125}I. This nomogram can be used to determine the conditions for the desired specific radioactivities for any unknown RNA. The *in vitro* iodinated RNA has been compared with *in vivo* labeled RNA for hybridization characteristics. Competition hybridization between ^{125}I-labeled chloroplast-rRNA and unlabeled pea (*Pisum sativum*) chloroplast-rRNA was identical to that found using [^{32}P]chloroplast-rRNA. Thermal stability of DNA-^{125}I-rRNA hybrids was similar to the thermal stability of DNA-[^{32}P]rRNA hybrids. The iodinated RNA was not found to have undergone any changes in its hydrogen-bonding properties.

Molecular hybridization between nucleic acids has been a powerful tool for studying the arrangement of genes and their location in DNA (1, 5, 18). DNA-RNA hybridization has provided investigators with quantitative estimates regarding the percentage of genome complementary to a given RNA sequence. Such a biochemical approach involving the molecular hybridization technique requires the isolation of reasonable quantities of nucleic acids with relatively high specific radioactivities. However, the inability to obtain nucleic acids with high specific radioactivities from eukaryotes in general, and from plants in particular, has been a perennial problem. This problem is further accentuated when one attempts to obtain adequately labeled plant organelle nucleic acids. The *in vitro* labeling methods using tritium exchange and methylation with tritium-labeled dimethyl sulfate (2, 3, 14) have never yielded satisfactory results under the most stringent experimental conditions. We have utilized the *in vitro* iodination method for nucleic acids originally developed by Commerford (4) and modified it to obtain reproducible hybridization results. Using this method, specific radioactivities of nucleic acids ranging from 10×10^4 to 10×10^6 cpm/μg have been achieved.

This paper described optimal *in vitro* iodination conditions for plant nucleic acids, provides a nomogram for determining desired specific radioactivity levels, and presents data to show that *in vitro* ^{125}I-labeled ribosomal (r-) RNA is comparable to *in vivo* ^{32}P-labeled rRNA with respect to their chemical characteristics in molecular hybridization. In order to demonstrate the applicability of this labeling technique, we have hybridized total chloroplast[3] ^{125}I-tRNA from peas (*Pisum sativum*) with pea Ct-DNA and shown that there are 30 to 40 tRNA genes in Ct-DNA. It may be noted that this information could not have been obtained readily by using the present *in vivo* labeling methods (16, 17). The utter simplicity of these iodination conditions and the extreme reproducibility in these results coupled with the apparent retention of the native properties of nucleic acids should make this method widely applicable to many other molecular problems involving plant development and differentiation.

MATERIALS AND METHODS

Chloroplast 4S RNA (tRNA) was isolated as follows. In a typical isolation, a total of 800 g of pea leaves (*Pisum sativum*) in 400-g lots of 18- to 20-day-old plants were homogenized in 1.5 liters of STM buffer (0.3 M sucrose, 50 mM tris-base [pH 7.8], 5 mM $MgCl_2$, 0.1 mM mercaptoethanol) and filtered through four layers of cheesecloth and two layers of Miracloth. The cell-free homogenate was then centrifuged at 1520*g* for 15 min in a Sorvall RC-2B. The pellet containing the crude chloroplast fraction was washed twice with STM buffer. The pelleted chloroplasts were then resuspended with TM buffer (5 mM $MgCl_2$, 25 mM tris-base [pH 7.8]) using 125 ml TM/800 g leaves. Twenty ml of 2% Lubrol was added, and after 15 min, the solution was centrifuged at 34,860*g* for 20 min. The supernatant was further centrifuged for 5 hr at 24,000 rpm in a Spinco SW 27 rotor. At the end of this last spin, the top two-thirds of the supernatant was collected by pipetting and to this solution 35 ml of EB buffer (25 mM tris-HCl-25 mM KCl-25 mM $MgCl_2$-2% SDS [pH 7.5]) was added. After mixing gently for 5 min at room temperature, the solution was extracted three times with phenol saturated with EB buffer. The RNA of the final aqueous phase was precipitated by adding 2 volumes of 95% ethanol and storing at −20 C overnight. The precipitated RNA was collected by centrifugation at 12,000*g* for 10 min and resuspended with 10 ml of TM buffer. Aliquots containing 60 to 80 $A_{260\ nm}$ units were loaded onto 5 to 20% TM buffer-sucrose gradients and centrifuged for approximately 40 hr at 24,000 rpm in a Spinco SW 27 rotor. The gradients were then fractionated and the 4S peaks pooled. The RNA was precipitated by adding 2 volumes of 95% ethanol and stored overnight at −20 C. The ethanol-precipitated 4S RNA was centrifuged as above and resuspended with 1 × SSC, using 2.5 ml 1 × SSC/800 g leaves starting material.

Cytoplasmic (Cyt-) rRNA and chloroplast (Ct-) rRNA were isolated from the purified 80S and 70S ribosomes, respectively, as described by Thomas and Tewari (19). Ct-DNA was obtained by the method of Kolodner and Tewari (20).

For *in vivo* labeling of plants, 6- to 7-day-old pea seedlings were pierced through the main stem with an absorbant cotton string. The seedlings were transplanted into vermiculite moistened with phosphate-free Hoagland medium (8). From a tube adjacent to the plant being labeled, 0.5 mCi ^{32}P as $H_3{}^{32}PO_4$ in 3 ml LB buffer (10 mM Na-acetate [pH 6]) was osmotically transfered through the string. The plants were labeled from 3 to 5 days. After this period, the plants were uprooted, the roots trimmed to 3- to 5-cm lengths, and allowed to grow 2 to 3 more days in [^{32}P]phosphate-free medium. RNAs were isolated from

[1] This research was supported by National Science Foundation Grant GB-20674.

[2] A preliminary report of this work was presented at the ASBC meeting in June 1974.

[3] Abbreviations: Ct-rRNA: chloroplast-rRNA; Cyt-rRNA: cytoplasmic-rRNA; HA: hydroxyapatite; SSC = 0.15 M sodium chloride-0.015 M sodium citrate.

these plants as described in the different sections above.

In vitro iodination was carried out with 0.74 to 1.47 $\times 10^{-4}$ M RNA (50–500 μg RNA in 2 ml final reaction volume). The RNA solution was made to 3.75 $\times 10^{-5}$ M with respect to KI and 0.2 M with respect to Na-acetate (pH 5). $Na^{125}I$ was added to a final concentration of 9.24 $\times 10^{-12}$ to 9.24 $\times 10^{-10}$ M (20–2000 μCi in the final 2-ml reaction volume). The reaction was started by the addition of 2.3 $\times 10^{-3}$ M $TlCl_3$ (K. & K. Laboratories; $TlCl_3$ can be kept frozen for about 2 months without affecting the extent of the reaction). This mixture was then incubated at 60 C for 30 min. At the end of this incubation, 0.29 M NH_4-acetate (pH 9) was added followed by 0.066 M Na_2SO_3. The mixture, now equilibrated at alkaline pH, was reincubated at 60 C for 20 min, passed through a Sephadex G-25 column or phenol extracted directly as described under "Results." We have taken only normal precautions for handling a volatile compound, and we had no incidence of flash-labeling of the thyroid gland. Hybridizations were carried out with DNA immobilized on nitrocellulose membrane filters as described before (19).

RESULTS

OPTIMUM CONDITIONS FOR *IN VITRO* IODINATION

Concentration of KI. It has been reported by Commerford (4) and others (6, 12) that the molar concentration of KI [KI] modulated the extent to which nucleic acids were iodinated. The most useful conditions for labeling RNA or DNA would be those which yielded maximal incorporation of radioactive nuclide with respect to the incorporation of the nonradioactive carrier nuclide. For the purpose of establishing this [KI] for plant RNAs, 25 μg of pea Cyt-rRNA/ml was iodinated with 90 μCi ^{125}I/ml in the presence of various [KI] (Table I). It can be seen from the table that the incorporation of ^{125}I into pea Cyt-rRNA varied with different [KI], reaching a maximum level at a [KI] of 3.75 $\times 10^{-5}$ M. The specific radioactivity (cpm/μg) obtained for the Cyt-rRNA at this [KI] was 374,000. However, when the [KI] was lowered below 3.75 $\times 10^{-5}$ M, the resulting specific radioactivity levels were also lower. For example, a specific radioactivity of 67,000 was obtained at a [KI] of 3.12 $\times 10^{-6}$ M. This reduction in the specific radioactivity level apparently indicated that the reaction conditions were submaximal at [KI] below 3.75 $\times 10^{-5}$ M. A decrease in specific radioactivity was also observed when the [KI] was present in excess of 3.75 $\times 10^{-5}$ M. This reduction resulted partly from the net decreased mol fraction of ^{125}I involved in the reaction and partly by the nature of the reaction. Under the reaction conditions which yielded maximal specific radioactivity level, about 21.4% of the total cytidylate content in pea Cyt-rRNA had been chemically iodinated. This calculation was based upon an average experimentally determined cytidylate content of 23.6% for plant Cyt-rRNAs (11, 21).

Table I. *Conditions for in Vitro Iodination*

KI	RNA	^{125}I	Specific Activity
(X10^{5}M)	(μg)	(μCi)	(cpm/μg)X10^{-3}
0.31	25	90	67
0.62	25	90	83
1.25	25	90	267
2.50	25	90	283
3.75	25	90	374
5.0	25	90	358
7.5	25	90	254
12.5	25	90	201
3.75	33	10	39
3.75	45	10	41
3.75	57	10	37
3.75	165	10	25
3.75	321	10	16
3.75	457	10	11
3.75	661	10	7
3.75	25	5	21
3.75	25	7.5	31
3.75	25	15	68
3.75	25	25	89
3.75	25	50	194
3.75	25	100	407

Concentration of $Na^{125}I$. The variations in ^{125}I concentrations at optimal [KI] would change the specific radioactivities proportionally with no effect on the over-all iodination levels. In order to test this relationship, experiments were carried out with 25 μg pea Cyt-rRNA/ml, 3.75 $\times 10^{-5}$ M KI at 60 C for 30 min using ^{125}I concentrations ranging from 5 μCi/ml to 100 μCi/ml. As can be seen from Table I, the specific radioactivity increased linearly with increasing concentrations of ^{125}I. This result clearly showed that until the ^{125}I concentration represented a significant proportion of the iodine in the reaction mixture, specific radioactivity levels would be directly proportional to the concentration of ^{125}I.

Concentration of RNA. In another series of experiments, the effect of increasing concentrations of Cyt-rRNA on the specific radioactivity levels was determined. Iodination was carried out with concentrations of RNA ranging from 30 μg/ml to 660 μg/ml in the presence of 3.75 $\times 10^{-5}$ M KI and 10 μCi ^{125}I/ml at 60 C for 30 min. It can be seen from Table I that the specific radioactivity levels decreased with increasing RNA concentrations.

Temperature. The optimum temperature for iodination was determined by analyzing the reaction rates at various temperatures. The reaction was carried out with 25 μg RNA, 90 μCi $Na^{125}I$, and 3.75 $\times 10^{-5}$ M KI. The time and temperature dependence of the iodination reaction can be seen in Figure 1. Near saturating levels of iodination were obtained at 52 C after 30 min of incubation. Raising the incubation temperature by an additional 8 C did not seem to effect significantly the level of radioactivity incorporated. It can be seen from Figure 1 that about 70% of the reaction was completed after 15 min and appeared to be essentially complete after 60 min. It would be of interest to note that milder iodination conditions would be desirable because of the susceptibility of rRNAs to breakdown as noted by some workers when iodinating at high temperatures for extended lengths of time (6, 7). However, all iodination reactions presented here were carried out at 60 C for 30 min. Under these experimental conditions, we have found that 28S rRNA does not get degraded below 12S.

Standardization of the [KI]/μg RNA·ml Ratio. The data from Table I pointed to the necessity of knowing the optimum concentrations of KI and RNA. Therefore, the data from a large number of different experiments were calculated and the [KI]/μg RNA·ml ratios plotted against specific radioactivities (Fig. 2). It can be seen from the figure that the optimal [KI]/μg RNA·ml ratio was about 14 to 20 $\times 10^{-7}$. This result made it possible to obtain desired specific radioactivity levels at any RNA concentration provided that the concentration of ^{125}I was proportionally increased with increasing concentrations of KI and RNA. That is to say, if the [KI] is doubled and the [KI]/μg RNA·ml ratio held constant, the concentration of ^{125}I must also be doubled in order to maintain the same specific radioactivity levels. From such a relationship, it was possible to construct the nomogram presented in Figure 3 correlating the specific radioactivity with concentrations of KI, RNA, and ^{125}I. The accuracy of the nomogram was examined by comparing the predicted specific radioactivities obtained experimentally. The results of these predictions and calculations are presented in Table II. The data clearly showed that the nomogram was accurate in determining the reaction conditions necessary to achieve desired specific radioactivity levels.

Iodination of tRNAs. The iodination conditions described above were used to iodinate Ct-tRNAs. The data obtained are given in Table III. It can be seen that the predicted specific radioactivity from the nomogram agrees reasonably well with the observed experimental values. The experiments were carried out

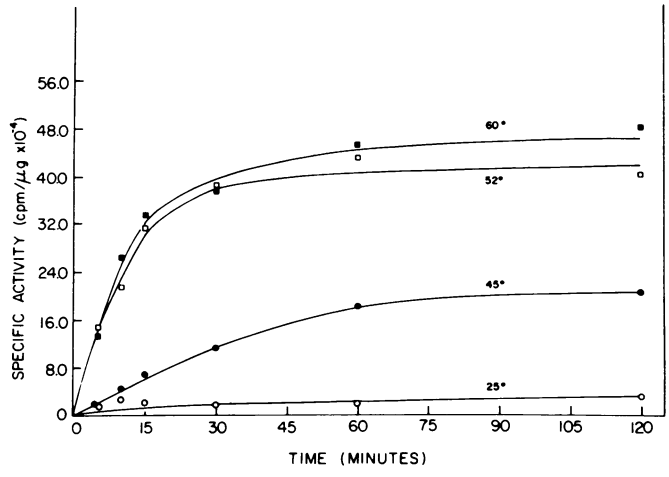

FIG. 1. Temperature dependence of iodination. Samples containing 3.75×10^{-5} M KI, 25 μg Cyt-rRNA/ml, and 90 μCi ^{125}I/ml were incubated at various temperatures and processed as described in text. (○), 25 C; (●), 45 C; (□), 52 C; (■), 60 C.

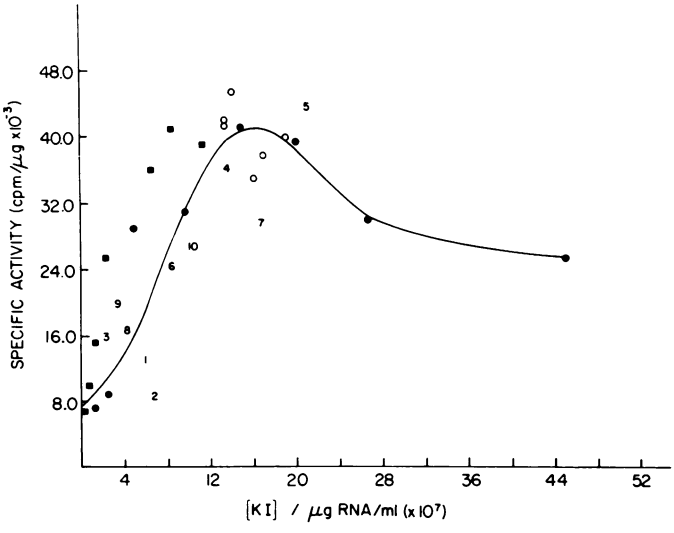

FIG. 2. Dependence of iodination of [KI]/μg RNA·ml ratio. Each point represents a reaction carried out as in Figure 1. Specific activities were normalized to the level that would be expected for 10 μCi ^{125}I/ml. (●), data from Figure 1; (○), data from Figure 3; (■), data from Figure 4; (Numbers), data from 10 independent experiments. (See Table I.)

at different concentrations of RNA and ^{125}I. The predicted results were always obtained. These data establish the reproducibility of the nomogram.

Purification of Iodinated RNA for Hybridization. There are three major complicating factors in utilizing ^{125}I-RNA for hybridization. One is the formation of a moderately stable 5-iodo-6 hydroxydihydropyrimidine. This reaction product was shown to be removed by a brief thermal treatment at alkaline pH by Commerford (4). The second problem is the apparent iodination of trace amounts of protein or other endogenous material present in the RNA preparations. This iodinated material caused the control filters to accrue an abnormally high number of counts during the hybridization experiments. This problem is the most important one in most hybridization experiments. The third problem is the presence of unreacted ^{125}I. This was effectively alleviated by the use of elution chromatography on Sephadex G-25 (4, 6). We have utilized this method for purifying iodinated RNA but ^{125}I-RNA prepared for hybridization experiments in this manner did not prove satisfactory although the same procedure has been found satisfactory by others. Therefore, it became necessary to purify the RNA further. This was accomplished by using one of the following procedures.

Proteins, RNA, and DNA can be effectively separated from each other through the use of Cs_2SO_4 buoyant density equilibrium centrifugation (15). The ^{125}I-RNA obtained from Sephadex G-25 column was precipitated with 95% ethanol, dissolved in a small volume of pH 5 buffer, and centrifuged in a Cs_2SO_4 gradient adjusted to a density of 1.55 g/cm^3 (15). It can be seen in Figure 4 that the RNA and covalently bound radioactivity track at the same density with a large amount of radioactivity (presumably the iodinated endogenous material) remaining at the top of the gradient. Because the ^{125}I-RNA purified in this manner formed very few nonspecific hybrids during hybridization experiments, it became clear that the iodinated material at the top of the Cs_2SO_4 gradient was responsible for the nonspecific hybridizations that had been observed.

In an effort to expedite the preparation of ^{125}I-RNA for hybridization experiments, the ^{125}I-RNA was extracted with phenol after iodination without using either column fractionation or Cs_2SO_4 gradients. The three phenol extractions were carried out as described under "Materials and Methods," and the ^{125}I-RNA precipitated with 2 volumes of 95% ethanol. The

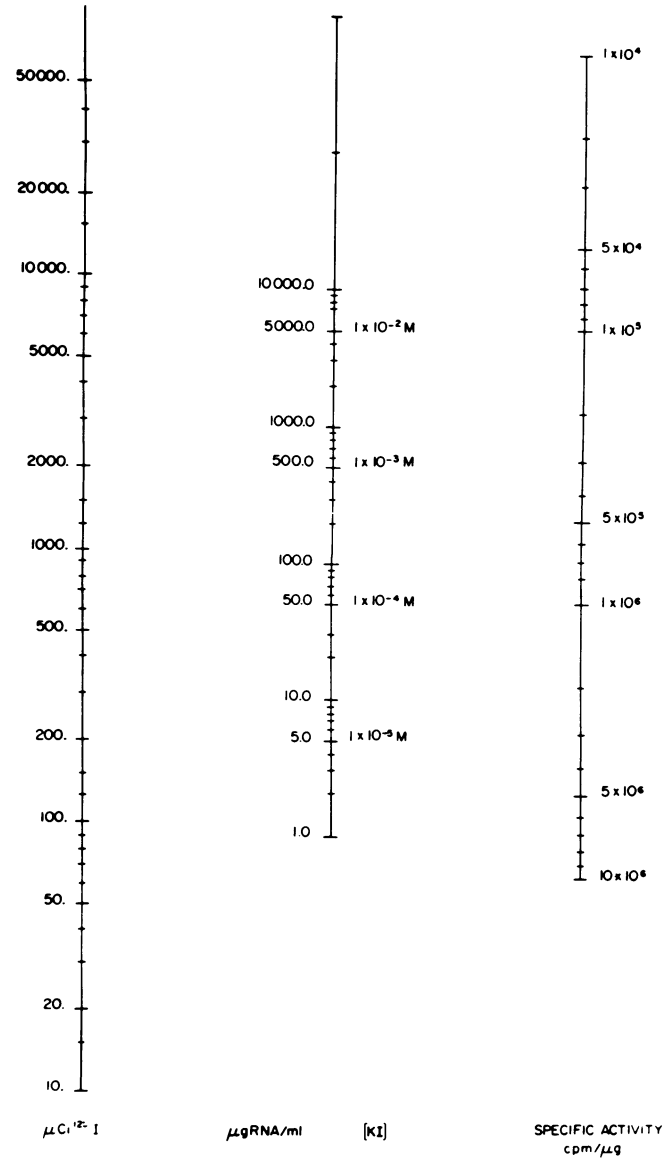

FIG. 3. Nomogram relating specific activity levels to concentrations of RNA, KI, and ^{125}I.

precipitated ^{125}I-RNA was successively washed with 65%, 75%, and 85% ethanol, redissolved in MGB buffer, and subsequently used for hybridization. ^{125}I-RNA prepared in this manner appeared to be relatively free of contaminating iodinated proteins and unreacted ^{125}I as demonstrated by the hybridization reactions.

The phenol extraction of the iodinated rRNA proved satisfactory whether the rRNA was obtained from 70S or 80S ribosomes. However, the iodinated tRNAs prepared as above were not found suitable in the hybridization experiments. In order to have a method applicable to RNAs irrespective of their source, we have devised the following procedure. The method has been described for tRNAs but can be used for any other RNAs. After the iodination reaction, the RNA was precipitated from the reaction mixture by adding 2 volumes of 95% ethanol and storing at −20 C for 2.5 hr. The precipitated RNA was centrifuged down at 12,000*g* for 10 min. The pellet was resuspended with 75% ethanol to help remove free iodine and then centrifuged again as above. The pellet was then resuspended with 0.5 ml of 0.01 M $NaPO_4$ buffer (0.005 M NaH_2PO_4, 0.005 M Na_2HPO_4 [pH 6.8]). The ^{125}I-tRNA solution was then layered onto a column of HA that had been previously equilibrated and thoroughly washed with 0.01 M $NaPO_4$ buffer. The HA column was then washed with 0.01 M $NaPO_4$ buffer until the A_{260} of the eluate was down to zero and the radioactivity of the fractions was negligible. The ^{125}I-tRNA was then eluted from the column using 0.25 M $NaPO_4$ buffer. This procedure gave 70 to 90% recovery of the initial amount of tRNA. This ^{125}I-tRNA was excellent for the hybridization experiments. In one experiment, the filters were incubated with 4.10^6 cpm of tRNAs. After overnight incubation, the control filters had absorbed only about 1000 counts. We have found that this procedure is the most reliable and simplest to carry out. Such a procedure has also been used by Shoulder *et al.* (13) in their experiments with tobacco necrosis virus. It has been our experience that extensive purification of RNA before iodination still results in iodinated RNA which is unsuitable in hybridization reactions.

Table II. *Comparison between Specific Activities Predicted by Nomogram and Those Obtained Experimentally*[1]

Concentration of RNA	Concentration of ^{125}I	[KI]/RNA	Experimental Specific Activity	Predicted Specific Activity
μg/ml	*μCi/ml*	Ratio[2] × 10^7	*cpm/μg*	*cpm/μg* × 10^{-3}
24.3	90.0	15.4	374,110	310
28.2	5.0	13.3	21,349	22
28.0	7.5	13.3	31,146	34
26.0	15.0	14.2	67,862	74
23.0	25.0	16.1	88,605	89
22.0	50.0	16.8	193,570	175
19.0	100.0	19.3	407,284	420
25.4	500.0	14.8	1,829,426	1,600
16.9	500.0	22.2	2,217,274	2,400
22.5	1000.0	16.7	2,985,533	3,300

[1] According to the nomogram presented in Figure 6.

[2] [KI] = 3.75 × 10^{-5} M; RNA = μg RNA/ml from column 1 of the table.

COMPARISON OF *IN VITRO* ^{125}I-LABELED AND *IN VIVO* ^{32}P-LABELED CT-RNA

Saturation Hybridization. In order to determine the effect of iodination on the base pairing property of the Ct-rRNA, saturation hybridization experiments were carried out between pea ^{125}I-Ct-rRNA and pea Ct-DNA. All hybridizations employed phenol-extracted ^{125}I-Ct-rRNA. *In vivo* ^{32}P-labeled Ct-rRNA (13,560 cpm/μg) and *in vitro* ^{125}I-labeled Ct-rRNA (1,641,000 cpm/μg) were compared for their rate of hybridization and saturation level at increasing μg RNA/μg DNA ratio (R/D ratio). At an R/D ratio of 1, 2.57% of Ct-DNA hybridized with ^{125}I-Ct-rRNA. At the same R/D ratio, 2.36% of Ct-DNA hybridized with [^{32}P]Ct-rRNA. Although the hybridization levels were the same, the net counts bound to the filter were 320 with [^{32}P]Ct-rRNA and 41,637 with ^{125}I-Ct-rRNA. At an R/D ratio of 5, the net counts bound were 552 and 61,928 for [^{32}P]Ct-rRNA and ^{125}I-Ct-rRNA, respectively. When the R/D ratio was increased to 10, the level of hybridization for ^{125}I-Ct-rRNA remained at a level of about 3.80% comparable to a level of 3.6% obtained with [^{32}P]Ct-rRNA. Figure 5 also shows that the rates of saturation are nearly equivalent when [^{32}P]Ct-rRNA and ^{125}I-Ct-rRNA are competed. The salient feature of this experiment is the fact that the specific radioactivities between the *in vivo* and *in vitro* labeled Ct-rRNAs are different by a factor of 100, but the levels of hybridization obtained are the same.

Competition Hybridization. In order to examine the reliability

Table III. *Iodination of tRNAs*

Concentration of tRNA	Concentration of ^{125}I	Experimental Specific Activity	Predicted Specific Activity
(μg/ml)	(μCi/ml)	(cpm/μg) X10^{-7}	(cpm/μg) X10^{-7}
5.7	10	74	130
5.7	20	200	270
5.7	50	557	680
5.7	100	1,040	1,300
15.2	10	64	50
15.2	20	208	93
15.2	50	546	240
15.2	100	995	500
6.9[a]	20	329	230
6.9	20	241	220

a) rRNA from 70S ribosomes

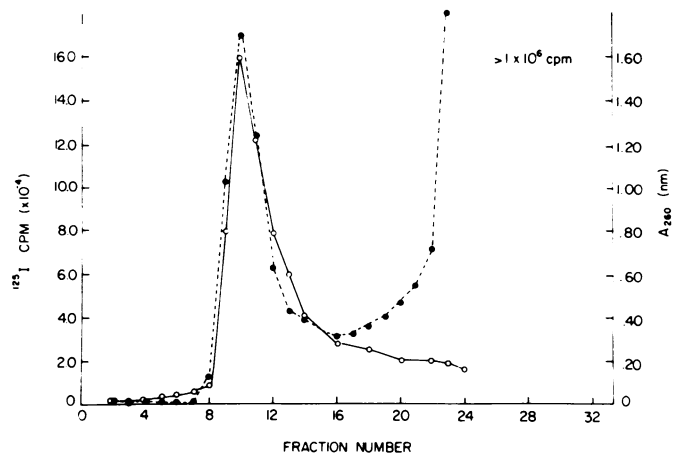

FIG. 4. Purification of ^{125}I-labeled pea Ct-rRNA in a Cs_2SO_4 density gradient. Pea Ct-rRNA was iodinated as described under "Materials and Methods" and passed through a Sephadex G-25 column. About 30 μg Ct-rRNA was mixed with 6.25 ml of 0.1 M Na-acetate buffer (pH 5), and 5.649 g Cs_2SO_4 was added to yield a final density of 1.55 g/cm^3. The gradient was centrifuged for 60 hr at 42,000 rpm in a Spinco fixed angle 50 rotor. Following centrifugation, the tube was pierced at the bottom, the fractions collected, and diluted to 0.5 ml. $A_{260\ nm}$ and counts were determined directly. O—O—, γ-radiation cpm; ●- -●- -, $A_{260\ nm}$.

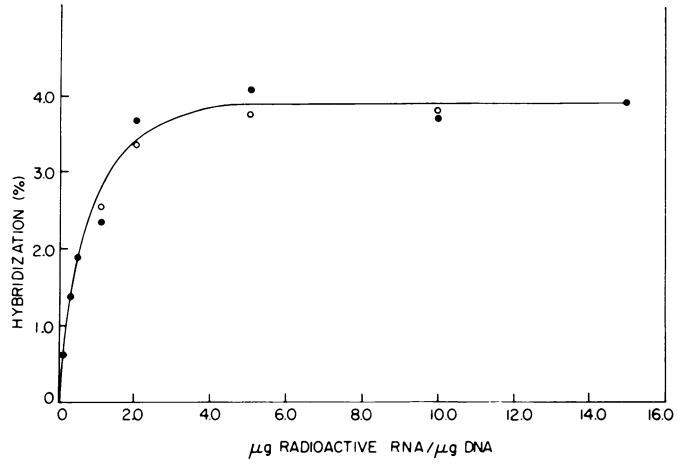

FIG. 5. Saturation hybridization between pea Ct-DNA and increasing amounts of *in vivo* ^{32}P-labeled or *in vitro* ^{125}I-labeled pea Ct-rRNA. Each experimental filter contained 1 μg Ct-DNA and was incubated along with a control filter containing 1.0 μg calf thymus DNA. (●), *in vivo* ^{32}P-labeled pea Ct-rRNA (13,560 cpm/μg); (○), *in vitro* ^{125}I-labeled pea Ct-rRNA (1,641,400 cpm/μg).

of the saturation hybridization data, competition hybridization experiments were utilized (Fig. 6). ^{125}I-Ct-rRNA (480,000 cpm/μg) was hybridized with 1 μg pea Ct-DNA at an R/D ratio of 3 with an initial hybridization level of 3.53%. Similarly, [^{32}P]Ct-rRNA (7920 cpm/μg) was hybridized with 2.5 μg pea Ct-DNA at an R/D ratio of 2.5 with an initial hybridization level of 4.73%. At a C/H ratio (μg unlabeled RNA/μg radioactive RNA) of 1, the hybridization level was reduced to 77.4% and 63.3%, respectively, for ^{125}I-Ct-rRNA and [^{32}P]Ct-rRNA. At a C/H ratio of 10, the hybridization levels were further reduced to 33.5% and 23.9%, respectively. The rates of competition between the differently labeled Ct-rRNAs are similar showing that iodinated RNA has the same characteristics as the naturally occuring RNA. In addition, the presence of unlabeled pea Cyt-rRNA with either of the labeled Ct-rRNAs showed no competition.

Thermal Stability. The final test as to the integrity of the DNA-rRNA duplexes formed during hybridization was that of their thermal stability. Thermal stability provides a direct measure as to the degree of microheterogeneity or mismatched base pairs. This criterion was of considerable importance here since these experiments were dealing with a homologous system but utilizing a chemically modified rRNA species. As can be seen from Figure 7, the melting profile of the Ct-DNA-^{125}I-Ct-rRNA hybrid was sharp and corresponded quite closely to the melting profile of Ct-DNA-[^{32}P]Ct-rRNA hybrid. The Tm (temperature of 50% dissociation) was 81 C for experiments involving ^{125}I-Ct-rRNA and 82 C for experiments involving [^{32}P]Ct-rRNA. For comparison, the thermal stability curve of ^{125}I-Ct-rRNA and calf thymus DNA was also presented in Figure 7. It can be clearly seen that amounts of nonspecific hybrids formed during these experiments were significantly removed at the incubation temperature of 65 C. These results are in agreement with those reported earlier (20). The ^{125}I label did not appear to have any discernible effects upon the Ct-DNA-Ct-rRNA hybrids. The iodinated RNA used in these experiments was stable to boiling.

tRNA Genes in Ct-DNA. We have used the iodinated Ct-tRNAs to determine the sequence homology with Ct-DNA. Saturation hybridization experiments have shown that there are 30 to 40 tRNA genes in the Ct-DNA. Competition experiments involving unlabeled Ct-tRNAs and Ct-^{125}I-tRNAs have shown that the tRNAs did not undergo any change in their hydrogen-bonding properties after *in vitro* iodination.

DISCUSSION

The results presented in this paper have shown that the maximum specific radioactivity levels resulting from *in vitro* iodination are directly dependent upon an optimal [KI]/μg RNA·ml ratio (*cf.* Fig. 2). The optimal [KI]/μg RNA·ml ratio was found to be between 14 and 20 $\times$ 10^{-7}. The optimal incubation conditions were 60 C for 30 min. When the optimal [KI]/μg RNA·ml ratio is maintained, it is possible to achieve desired specific radioactivity at any RNA concentration by varying the concentration of ^{125}I. This relationship is seen in the nomogram presented in Figure 3. It is clear that the experimentally obtained specific radioactivities are very near to the specific radioactivities predicted by the nomogram. The iodination conditions were worked out with rRNA. When the nomogram was used for

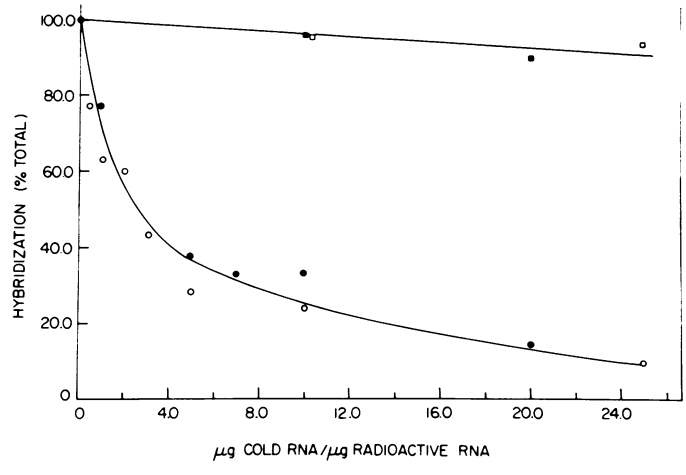

FIG. 6. Competition hybridization between *in vivo* ^{32}P-labeled or *in vitro* ^{125}I-labeled pea Ct-rRNA with unlabeled pea Ct-rRNA. ^{32}P-Labeled Ct-rRNA (7920 cpm/μg) was hybridized to 2.5 μg pea Ct-DNA at an R/D ratio of 2.5 in the presence of increasing amounts of (○), unlabeled pea Ct-rRNA; (□), unlabeled pea Cyt-rRNA. ^{125}I-Labeled Ct-rRNA (480,600 cpm/μg) was hybridized to 1 μg pea Ct-DNA at an R/D ratio of 3 in the presence of increasing amounts of (●), unlabeled pea Ct-rRNA; (■), unlabeled pea Cyt-rRNA.

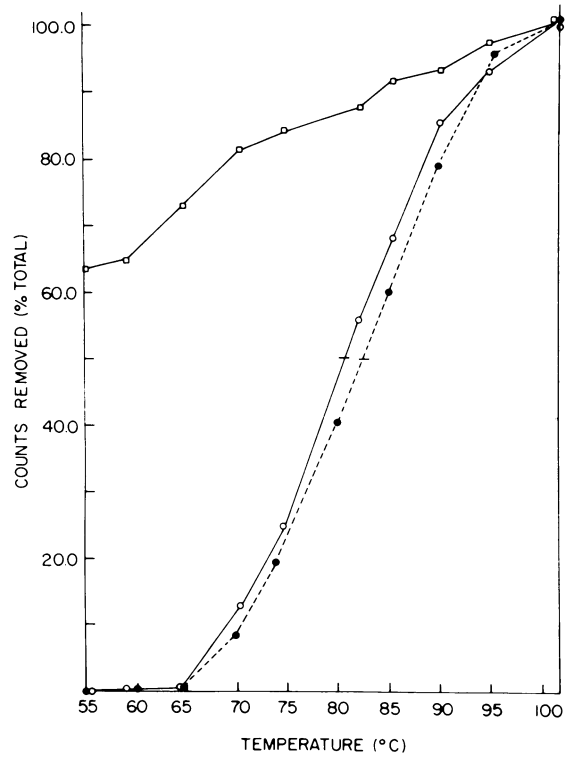

FIG. 7. Thermal stability of pea Ct-DNA and ^{32}P-labeled or ^{125}I-labeled Ct-rRNA hybrids. ●- -●- -, ^{32}P-labeled pea Ct-rRNA (5380 cpm/μg) was hybridized to 5 μg pea Ct-DNA in R/D ratios of 2. ○—○—, ^{125}I-labeled pea Ct-rRNA (751,300 cpm/μg) was hybridized to 1 μg pea Ct-DNA at an R/D ratio of 5. □—□—, ^{125}I-labeled pea Ct-DNA (751,300 cpm/μg) was hybridized to 1 μg calf thymus DNA control filter at an R/D ratio of 5.

tRNAs, the predicted values were found to be reasonably close to those obtained experimentally.

In order to utilize iodinated RNA for hybridization experiments, it was necessary to examine closely the effect that such a chemical modification might have on the over-all hybridizability of the ^{125}I-RNA. It became apparent from the initial hybridization experiments that in addition to the labeling of RNA, some other material, presumably trace amounts of protein, also became labeled. This was reflected in hybridization blanks which contained 75 to 95% of the counts found on the experimental filters.

In an effort to obtain RNA preparations which exhibited low controls in hybridization experiments, methods of additional purification were examined. The first method involved the purification of ^{125}I-Ct-rRNA in Cs_2SO_4 density gradients. Using this ^{125}I-Ct-rRNA, the counts bound to the control filter dropped significantly to a level of around 10% of the experimental filter. In order to expedite and simplify the procedure further, a second method was used. This involved the extraction of iodinated RNA with phenol followed by alcohol precipitation. The results obtained from such a purified ^{125}I-RNA proved to be even better in that the counts bound to the control filter were lowered to a level of about 7% of the experimental filters. The third method involved the purification of ^{125}I-tRNAs on HA. This method is of choice where low hybridization values are expected, because it gives much lower nonspecific binding on the filter than the other two methods.

In order to demonstrate the usefulness of the chemically modified ^{125}I-Ct-rRNA in hybridization, it was necessary to investigate three important criteria: the ability of the ^{125}I-Ct-rRNA to saturate a homologous Ct-DNA to a constant level; competition rates between ^{125}I-Ct-rRNA and [^{32}P]Ct-rRNA with nonlabeled rRNA; and the extent of accurate base pairing between the Ct-DNA and ^{125}I-Ct-rRNA. Therefore, pea ^{125}I-Ct-rRNA and pea [^{32}P]Ct-rRNA were hybridized with pea Ct-DNA, and the rates of hybridization and the saturation levels compared. The results clearly showed that the hybridization properties of the ^{125}I-Ct-rRNA were nearly identical with those of the [^{32}P]Ct-rRNA. For example, [^{32}P]Ct-rRNA saturated 3.8% of the Ct-DNA. In similar experiments, ^{125}I-Ct-rRNA saturated 3.67% of the Ct-DNA. In competition experiments, ^{125}I-Ct-rRNA and [^{32}P]Ct-rRNA were hybridized at a constant R/D ratio and competed with increasing amounts of nonlabeled pea Ct-rRNA or pea Cyt-rRNA. In such experiments (Fig. 6), the base sequences of ^{125}I-Ct-rRNA and [^{32}P]Ct-rRNA were indistinguishable when competed with nonlabeled Ct-rRNA. Furthermore, Cyt-rRNA did not compete with ^{125}I-Ct-rRNA. The most critical test as to the accuracy and specificity of the DNA-RNA hybridization is the criteria of thermal stability of the hybrids (9, 10). It can be seen from Figure 7 that the melting profile of Ct-DNA-^{125}I-Ct-rRNA is quite sharp. The Tm of Ct-DNA-^{125}I-Ct-rRNA hybrid is 81 C compared to a Tm of 82 C for Ct-DNA-[^{32}P]Ct-rRNA hybrid. Furthermore, the Ct-DNA-^{125}I-Ct-rRNA hybrid melts as a homogeneous component without suggestion of heterogeneity. The results are again in agreement with those obtained with the Ct-DNA-[^{32}P]Ct-rRNA hybrid.

In conclusion, RNA can be labeled to a high specific activity *in vitro* by ^{125}I. The *in vitro* labeled RNA has the same chemical characteristics in hybridization as the *in vivo* labeled RNA.

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