Isolation of 5-Fluorouracil-Containing 5S Ribonucleic Acid from *Escherichia coli**

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ABSTRACT: Low molecular weight ribosomal ribonucleic acid isolated from *Escherichia coli* B grown in the presence of 5-fluorouracil consists of a mixture of normal and 5-fluorouracil-containing 5S ribonucleic acid molecules. This mixture, which chromatographs as a single component on Sephadex G-100, may be separated into the normal and 5-fluorouracil-containing molecules by DEAE-cellulose column chromatography in the presence of 0.02 M Tris-HCl (pH 8.9) and a concave upward NaCl gradient. Recovered 5-fluorouracil-containing 5S ribonucleic acid has been shown to have up to 83% of the uracil residues replaced by 5-fluorouracil. None of the other bases are effected. This replacement is comparable with that found in transfer ribonucleic acid. Recoveries were greater than 85%. Rechromatography of the 5-fluorouracil-containing 5S ribonucleic acid on DEAE-cellulose did not reveal the presence of any contaminating normal 5S ribonucleic acid. This suggests that complete replacement of the uracil by 5-fluorouracil is not possible. Sephadex G-100 chromatography indicated that no appreciable amounts of transfer ribonucleic acid were present in this fraction and that no apparent aggregation occurred during the DEAE-cellulose chromatography.

Recent results have indicated that in addition to uracil, FU[1] replaces a number of structurally related minor pyrimidine components in tRNA from *Escherichia coli*. These include pseudouridine (Andoh and Chargaff, 1965; Lowrie and Bergquist, 1968; Johnson *et al.*, 1969), ribothymidine (Lowrie and Bergquist, 1968; Johnson *et al.*, 1969; Baliga *et al.*, 1969), 4-thiouridine (Kaiser, 1969b), and 5,6-dihydrouridine (Kaiser *et al.*, 1969). The biological role of these minor components is unknown. A careful examination of normal and FU-containing tRNA structure and function may reveal differences and lead to possible insights into the function of these minor bases. One limitation of this approach is the unknown effect of the substitution of FU for uracil in FU-containing tRNA, as pointed out by Johnson *et al.* (1969).

An effort to examine the importance of this single substitution of uracil by FU, has prompted the isolation of low molecular weight (5 S) rRNA rich in FU. This species of RNA appears highly suitable, since it is only slightly larger than tRNA and does *not* contain any methylated or other minor base components (Sanger *et al.*, 1968). Its nucleotide composition is also similar to the major components found in the mixture of tRNAs from *E. coli*. In addition, it is homogeneous, except for possible minor differences (Sanger *et al.*, 1968), and perhaps quite similar to tRNA in secondary and tertiary structure (Boedtker and Kelling, 1967; Cantor, 1968; Aubert *et al.*, 1968b).

Johnson *et al.* (1969) recently reported the FU replaces uracil in the 5S RNA species from *E. coli* and that the molecule remains attached to the ribonucleoprotein particles. The material which they isolated had 35% of the uracil replaced by FU.

This report describes the isolation of 5S RNA having up to 83% of the uracil residues replaced by FU. There appears to be no normal 5S RNA remaining in the sample and no appreciable amount of contaminating tRNA.

Materials and Methods

Bacterial Growth. These studies were carried out with *E. coli* B and grown as previously described (Kaiser, 1969a).

Preparation of 5S RNA. Both normal and FU-5S RNAs were isolated from bacterial cells along with tRNA by direct phenol extraction as described earlier (Kaiser, 1969a). The majority of the contaminating tRNA was removed by repeated chromatography over Sephadex G-75 and G-100 at room temperature and neutral pH (Schleich and Goldstein, 1964).

Preparation and Operation of DEAE-cellulose Columns. DEAE-cellulose with a capacity of 1.0 mequiv/g (Whatman DE-32, microgranular) was used. Precycling of the exchanger, equilibration, and pouring of the columns were carried out as suggested by the manufacturer (Whatman Technical Bulletin IE 2). Chromatography was carried out at room temperature (*ca.* 27°) on 1 × 40 cm columns at a flow rate of 10–20 ml/hr. A concave upward gradient of NaCl buffered with 0.02 M Tris-HCl (pH 8.9) was used in all elutions. This gradient was generated by level, cylindrical bottles of dissimilar size open to the atmosphere. The reservoir (3.5 cm in diameter, containing 75 ml of 0.70 M NaCl) was connected to the mixing vessel (6.0 cm in diameter, containing 225 ml of 0.35 M NaCl) by a small diameter siphon. The salt concentrations represented in the figures were determined by periodically measuring the refractive indexes of various tubes with an Abbe 3-L refractometer (Bausch & Lomb).

* From the Division of Biochemistry, University of Wyoming, Laramie, Wyoming 82070. *Received September 26, 1969.* Published with approval of the Director, Wyoming Experiment Station, as Journal Paper No. 400. This paper is the fourth in a series dealing with fluorouracil-containing RNA.

[1] The following abbreviations are used: FU, 5-fluorouracil; FU-5S RNA, 5S RNA containing FU; A_{260} unit, a unit of material which in a volume of 1 ml will have an absorbance of 1 at 260 mμ when measured in a cell of 1-cm path length.

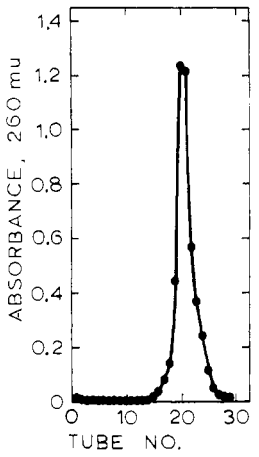

FIGURE 1: Gel filtration of FU-5S RNA on Sephadex G-100. FU-5S RNA (21 A_{260} units) was applied to a column of Sephadex (0.9 × 233 cm) and collected as described in Methods.

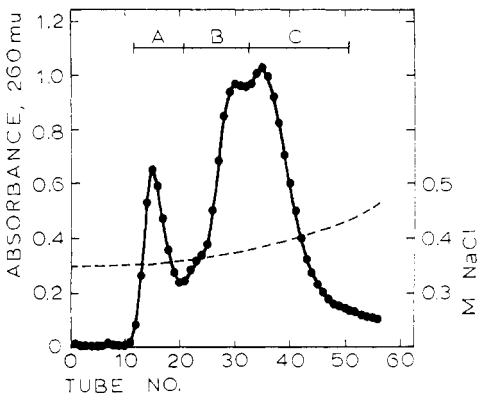

FIGURE 2: DEAE-cellulose column chromatography of FU-5S RNA. An aliquot of the FU-5S RNA (105 A_{260} units) chromatographed in Figure 1 was applied to a column of DEAE-cellulose (1 × 40 cm) and eluted with a concave upward NaCl gradient buffered with 0.02 M Tris-HCl (pH 8.9). The indicated fractions were pooled and collected (see Methods). Absorbance (●—●) and NaCl concentrations (– – – –) were measured as described in the text.

The absorbancies of the fractions were determined at 260 mμ in either a Beckman DU or Hitachi Perkin-Elmer Model 139 spectrophotometer, using silica cells with a path length of 1 cm. Recoveries of A_{260}-absorbing material were greater than 85%.

RNA was recovered by ethanol precipitation as described (Kaiser, 1969a). About 4.3 ml was collected per tube.

Sephadex Chromatography. All Sephadex G-100 chromatography was carried out at room temperature on columns (0.9 × 233 cm) equilibrated with 0.02 M Tris-HCl (pH 8.9)–0.50 M NaCl buffer unless otherwise noted. Flow rates were *ca.* 10 ml/hr and the RNA was collected by ethanol precipitation as described for the DEAE-cellulose columns. Approximately 4.3 ml was collected per tube. Recoveries of A_{260}-absorbing material were around 95%.

Hydrolysis and Nucleotide Analysis. Hydrolysis and nucleotide analyses of the 5S RNA samples were essentially as described (Kaiser, 1969a), except absorbancies were measured in a Beckman DU monochrometer with a microaperature plate, 1200-μl silica cuvets (1-cm path length), and a Gilford Model 222 photometer.

Materials. 5-Fluorouracil was a gift from Dr. W. E. Scott of Hoffman-LaRoche, Inc.; Tris (Ultra Pure) was obtained from Mann Research Laboratories, New York; all other materials were as previously described (Kaiser, 1969a).

Results

Chromatography of FU-5S RNA. After several passages over Sephadex G-75 or G-100, the FU-5S RNA used in these studies exhibited one peak upon Sephadex G-100 chromatography (Figure 1). The trailing edge of this peak was slightly skewed, indicating a small amount of lower molecular weight material might still be present. Chromatography on 0.9 × 233 cm columns of Sephadex G-75 and G-100 using a neutral buffer (0.01 M potassium cacodylate (pH 7.0)–0.30 M KCl), gave essentially the same elution profile as shown in Figure 1.

When this sample was applied to a column of DEAE-cellulose and eluted with a concave NaCl gradient, buffered at a pH of 8.9, the elution profile shown in Figure 2 was obtained. Although this sample of FU-5S RNA chromatographed essentially as a single component on Sephadex (Figure 1), it was resolved into several peaks on DEAE-cellulose. The material in fractions A, B, and C represents 17, 35, and 45%, respectively, of the ultraviolet-absorbing material recovered. On other runs, fraction B was usually less prominent and was represented only by a shoulder on fraction C.

Three separate 5S RNA samples (A, B, and C) were pooled from the run shown in Figure 2, and collected by ethanol precipitation and centrifugation. Each of these fractions were then rechromatographed on DEAE-cellulose exactly as the original 5S RNA sample shown in Figure 2. Although not shown, the eluting salt gradient was the same in these runs as in Figure 2. The elution profiles for each of these fractions is shown in Figure 3. Sample A shows the majority of ultraviolet-absorbing material to elute before tube 20, although there is considerable trailing of this fraction. Sample B shows a complex elution profile. Only 20% of the recovered ultraviolet-absorbing material eluted between tubes 21 and 32, where this fraction was originally collected (Figure 2). Most of the material (70%) eluted later, and most likely is similar to sample C. Nearly 90% of the final fraction collected in Figure 2 (sample C), eluted in the same position upon rechromatography, *i.e.*, between tubes 32 and 52. Neither sample B nor C had any appreciable absorbance eluting prior to tube 20 (that region corresponding to sample A).

The possibility existed that aggregation or a change in the conformation (Aubert *et al.*, 1968b) of 5S RNA was occurring during chromatography on DEAE-cellulose and was responsible for the trailing shown in Figure 3A and the somewhat unusual looking profile obtained for sample B upon rechromatography (Figure 3B). To investigate this possibility, samples corresponding to A, B, and C were collected from a DEAE-cellulose chromatography run carried out exactly as the one shown in Figure 2. Each of these samples were then chromatographed on Sephadex G-100 at pH 8.9 in the presence of 0.50 M NaCl. No aggregation or conforma-

TABLE I: Base Composition of Normal and FU-5S RNAs.[a]

Nucleotide	Normal 5 S[b]	FU-5 S[c]	Sample[d,e] A	Sample[d] B	Sample[d] C
C	30.0	27.0 ± 0.40	28.2 ± 0.16	26.9 ± 0.33	26.9 ± 0.26
G	34.2	35.7 ± 0.65	36.2 ± 0.04	36.4 ± 0.87	35.4 ± 0.39
A	19.2	19.3 ± 0.18	18.4 ± 0.26	19.8 ± 0.52	20.3 ± 0.17
U	16.7	6.17 ± 0.56	17.3 ± 0.50	6.26 ± 0.28	2.92 ± 0.18
FU		11.8 ± 0.36		10.5 ± 0.56	14.4 ± 0.27
$\frac{FU}{FU+U} \times 100$	0	66	0	63	83

[a] Analyses were carried out as described in Methods. Results expressed as mole percentage plus or minus standard deviation. [b] Base composition calculated from the known sequence of 5S RNA from *E. coli* (Sanger *et al.*, 1968). [c] FU-5S RNA sample *before* DEAE-cellulose chromatography. [d] Samples correspond to those pooled and recovered after DEAE-cellulose, followed by Sephadex G-100 chromatography. [e] Limit of detection for FU, was estimated to be <0.5 mole %.

tion change leading to a more expanded structure was apparent in the samples, as determined by Sephadex chromatography.

Chromatography of Normal 5S RNA. The normal 5S RNA exhibited a single, symmetrical peak on Sephadex G-100 columns both before and after passage over DEAE-cellulose. Again there was no evidence of either aggregation or a stable conformational change occurring during chromatography and no evidence at all of tRNA contamination in this sample.

When the normal 5S RNA was passed over DEAE-cellulose the profile shown in Figure 4 was obtained. The majority of the material eluted in the same position as sample A in Figure 2. This indicated that sample A consisted primarily of normal 5S RNA as may have been suspected from earlier work with normal and FU-containing tRNA (Kaiser, 1969a). The reason for the small shoulder on the trailing edge of the normal 5S RNA profile (Figure 4) is not clear and will be considered again in the Discussion.

Base Analysis of 5S RNA. The FU content of the FU-5S RNA before and after DEAE-cellulose chromatography is shown in Table I. The FU-5S RNA sample represents material *before* DEAE-cellulose chromatography. This particular sample was collected after two additional passages (not shown) over Sephadex G-75, to ensure elimination of all tRNA. Samples A, B, and C (Table I) correspond to the fractions obtained after DEAE-cellulose chromatography (Figure 2). These samples were passed over Sephadex G-75 following the DEAE-cellulose step to ensure complete elimination of any tRNA, before the base analyses were carried out.

Discussion

When 5S RNA is isolated from *E. coli* B, previously treated with FU, extensive replacement of the uracil residues by FU is noted. Up to about 65% replacement has been obtained. The mole percentages of the other major bases remains essentially unchanged as shown in Table I and as

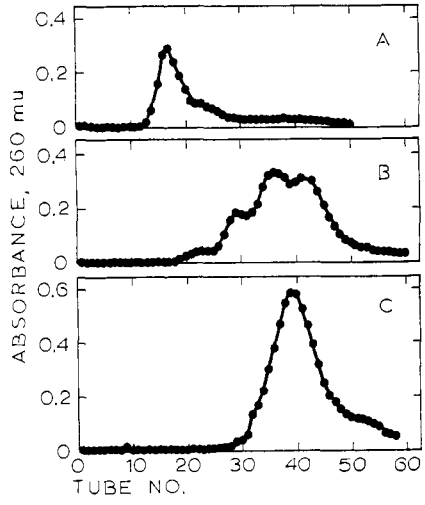

FIGURE 3: Rechromatography on DEAE-cellulose of pooled fractions A, B, and C, from Figure 2. Each of the recovered fractions were rerun separately exactly as described for Figure 2. Figures A, B, and C correspond to fractions A, B, and C, respectively.

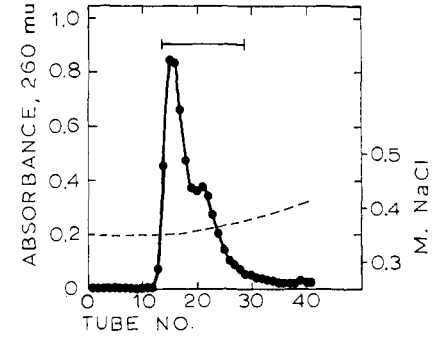

FIGURE 4: DEAE-cellulose chromatography of normal 5S RNA. A fraction (29 A_{260} units) of normal 5S RNA was applied to a 1 × 40 cm DEAE-cellulose column and eluted as discussed in Methods. Absorbance (●—●) and NaCl concentrations (----) were measured as described in the text.

previously reported by Johnson *et al.* (1969). These authors indicated, however, that the uracil replacement by FU was only 35%. The difference between their data and those reported here may be the result of: (1) the length of exposure of *E. coli* to FU (2 *vs.* 3 hr) and/or (2) the method of RNA preparation.

The FU-5S RNA sample passed over Sephadex G-100 in Figure 1, contained about 20% normal 5S RNA. No separation between the normal and FU-containing material was apparent. Similar results were obtained on Sephadex G-75. A comparison (not shown) of the gel filtration profiles of normal and FU-5S RNA (having 83% of the uracils replaced by FU) also revealed no differences, with the peak tubes of both samples being identical.

When the FU-5S RNA sample chromatographed in Figure 1 was applied to DEAE-cellulose and eluted as described, several components were apparent (Figure 2). From previous experience with FU-containing tRNA and results with normal 5S RNA, fraction A (Figure 2) was suspected to be normal 5S RNA. This was confirmed when *no* FU was detected in this fraction upon nucleotide analysis (Table I). Fractions B and C were found to have 63 and 83% of their uracils replaced by FU, respectively. Fraction B is probably composed of 5S RNA molecules with varying amounts of the uracil residues replaced by FU. This was suggested by the 280 mμ:260 mμ ratio (not shown) which increases in going from fraction A to fraction C (Figure 2). The majority of this increase occurs over the first two-thirds of fraction B, indicating an increase in FU content. The ratio remains relatively constant over fraction A, C, and the latter part of fraction B. Base analyses of fractions A, B, and C show a progressive increase in the percentage of uracil replaced by FU in going from 0 to 63 to 83%, respectively. The extent of uracil replacement by FU in the 5S RNA contained in fraction C is comparable with that obtained under similar conditions for FU-containing tRNA (see Kaiser, 1969a).

Rechromatography on DEAE-cellulose, of fractions A, B, and C from Figure 2 are shown in Figure 3. Although fraction A shows a considerable amount of trailing, no FU could be detected in this fraction (Table I). Fraction B elutes as a complex pattern, and supports the earlier discussion which indicates that it represents a mixture of 5S RNAs containing various amounts of FU. As indicated earlier over 70% of fraction B rechromatographed as fraction C. This would suggest that the majority of this middle fraction closely resembles fraction C, or is identical with it. Fraction C rechromatographed very nearly the same as it did initially. There was no evidence for *any* normal 5S RNA being present in fraction C and possibly only a trace in fraction B. As with FU-containing tRNA, complete replacement of uracil by FU does not seem to occur.

Recoveries from DEAE-cellulose columns were greater than 85%. As much as 14 mg of FU-5S RNA was chromatographed on a 1 × 40 cm column of DEAE-cellulose.

Normal 5S RNA chromatographed as a single component on Sephadex G-100 *before* and *after* DEAE-cellulose chromatography. It was surprising to find that during some DEAE-cellulose chromatography runs more than one component appeared to be present (Figure 4). On other passages normal 5S RNA gave only one peak, with only a slight indication of a shoulder on the trailing edge. The reason for the trailing component in Figure 4 is unclear, although it may represent an unstable conformation change which takes place during the chromatography on DEAE-cellulose. If this occurs, it apparently reverts back to its normal molecular dimensions before rechromatography on Sephadex. Reversible conformational changes have been detected before with normal 5S RNA (Aubert *et al.*, 1968a,b).

Of the major bases, only uracil is replaced by FU in all species of RNA (Horowitz and Chargaff, 1959; Andoh and Chargaff, 1965; Lowrie and Bergquist, 1968; Johnson *et al.*, 1969). In a molecule such as 5S RNA, which does not contain any minor bases, *only* uracil is replaced. In tRNA from *E. coli* however, minor nucleosides structurally related to uridine, including pseudouridine, ribothymidine, 4-thiouridine, and 5,6-dihydrouridine are grossly deficient in FU-containing tRNAs.

The reduced levels of pseudouridine and ribothymidine in FU-containing tRNA prompted Johnson *et al.* (1969) to speculate on the usefulness of FU-containing tRNA in examining the role of the two minor nucleosides. They further indicated, however, that any experiments designed to do this would be complicated by the unknown effects of the substitution of FU for uracil and by the presence of normal tRNA along with the FU-containing species in the isolated tRNA.

A careful examination and comparison of the secondary and tertiary structure of FU-5S RNA with normal 5S RNA should reveal any structural changes brought about by the replacement of uracil with FU. These findings could then be hopefully used in the study of FU-containing tRNAs. Gross differences in the structures of either FU-containing transfer or 5S RNAs from their normal components, could possibly be attributed to one or more of the deficient minor bases. Both transfer and 5S RNAs containing FU have been isolated in a form having less than 20% of their usual uracil content. No *normal* transfer or 5S RNA contamination is detectable in these samples.

Studies on the extent of base pairing in normal 5S RNA from *E. coli* (Boedtker and Kelling, 1967; Cantor, 1968) have indicated that the structure of this species of RNA may closely resemble the cloverleaf model proposed for tRNA (Holley *et al.*, 1965) and contain several short helical sections. These observations further suggest, that the findings with 5S RNA may be most useful in explaining whether the FU replacement of either uracil or the minor base components in tRNA is responsible for the observed functional and structural changes.

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Calf Intestine Adenosine Deaminase. Substrate Specificity*

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ABSTRACT: Substitutions can be made in the 8 position of the purine nucleus to produce compounds which still retain their ability to be bound to the "nonspecific" adenosine deaminase, isolated from calf intestine. Such substitutions must be of a limited nature. Thus, while 8-amino-2′-deoxyadenosine is deaminated, the 8-*N*-methylamino derivative is not bound to the enzyme. Furthermore, while both N^1-methyladenosine (IV) and 8-oxoadenosine (V) are substrates for the enzyme the latter is deaminated at a rate that is 3000 times slower than IV. 8-Aminoadenosine and 8-azaadenosine were also found to be substrates for the enzyme. The K_m values for these alternate substrates varied within a relatively narrow range (2.35–13.7 $\times$ 10^{-5}) while the V_{max} values varied over approximately a 6000-fold range. The results of these substrate studies are discussed in relation to the steric and electronic properties of the purine nucleoside analogs studied. All of the alternate substrates were also competitive inhibitors of the deamination of adenosine and in addition several of the 2-substituted adenosine and deoxyadenosine derivatives that were not deaminated were found to be competitive inhibitors of the enzyme. 2-Aminopurine riboside was the best inhibitor and had a K_i value of 4.0 $\times$ 10^{-6}.

The study of enzymes responsible for the deamination of purine and pyrimidine nucleosides has both a theoretical and practical interest. On the one hand, a great number of potentially useful chemotherapeutic agents have been synthesized which possess the nucleoside structure, only to find that while the compounds were effective antimetabolites *in vitro* or in tissue culture, they were rapidly inactivated by deamination *in vivo*. Cytosine arabinoside and adenine arabinoside have been found to have antitumor and antiviral activity (Privat de Garilhe and De Rudder, 1964; Buthala, 1964) but both are deaminated to relatively inactive compounds by the respective cytidine and adenosine deaminases (Pizer and Cohen, 1960; Hubert-Habart and Cohen, 1962). A thorough understanding of the effect of substrate modification on enzyme activity could afford the knowledge necessary to design effective chemotherapeutic agents that would either be resistant to, or inhibitors of the mammalian deaminases.

The availability of a mammalian enzyme in relatively pure form capable of deaminating adenosine as well as an active program in the synthesis of purine nucleoside antimetabolites of potential chemotherapeutic value, led us to study what effects ring modification of adenosine had on the substrate specificity of calf intestine adenosine deaminase.

Previous studies with this enzyme, have shown that various modifications can be made in the carbohydrate moiety of the adenosine molecule to produce analogs of adenosine that still retain either all or part of their ability to be bound to the enzyme (Bloch *et al.*, 1967; Frederiksen, 1966). A number of ring-modified compounds have been studied as substrates and/or inhibitors of the enzyme. It has been shown (Schaeffer and Odin, 1965; Schaeffer and Vince, 1968; Schaeffer and Bhargava, 1965; Schaeffer *et al.*, 1965) that replacement of the ribose moiety at the 9 position with various alkyl, hydroxyalkyl, and aralkyl functions produced compounds that would bind to the enzyme. Wolfenden has recently demonstrated that replacement of the ribose moiety at N-9 with H (adenine) does not completely abolish the ability of the compound to bind to the enzyme as a substrate (Wolfenden *et al.*, 1969). There is however a rather large diminution of the rate of reaction ($^1/_{30,000}$), although the K_m of adenine is not drastically different from adenosine's. Several workers have shown that various substitutions can be made in the 2 and 6 position of the purine nucleus while still maintaining the ability of the compounds to bind to the enzyme (Chassy and Suhadolnik, 1967; Cory and Suhadolnik, 1965; Baer *et al.*, 1968; Frederiksen, 1966). Further, 1-methyladenosine (Wolfenden *et al.*, 1969) and *N*-3-(β-D-ribofuranosyl)-6-aminopurine (Wolfenden *et al.*, 1969) are also deaminated by the enzyme.

It is the general purpose of our program to study how structural modifications within the purine nucleus of the

* From ICN Nucleic Acid Research Institute, International Chemical & Nuclear Corporation, Irvine, California 92664. *Received September 30, 1969.*