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# Characterization of the RNA Synthesizing Activity of Isolated Kidney Nuclei

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*Abstract*

The limiting factor in RNA synthesis by isolated kidney nuclei is RNA nucleotidyltransferase at high salt concentrations but at low salt concentrations template availability becomes limiting. α-Amanitin inhibits 85% of the activity at high salt concentrations but only 20–50% of the activity at low salt concentrations. Exogenous DNA is utilized at low salt concentrations [up to 0·1M $(NH_4)_2SO_4$] but not at high salt concentrations. The effect of increasing salt concentration is mainly to cause an increase in the length of chains synthesized. Initiation rates are not increased by high salt concentrations. The apparent $K_m$ for UTP is 8–10 μM at high salt concentrations, indicating that assays performed at low UTP concentrations are likely to give inaccurate results. The activation energy for the reaction at low salt concentration is less than that for the reaction at high salt concentration. The RNA synthesizing capacity of kidney nuclei is dependent on the method of isolation, and preparation by a modification of the Chauveau method (Chauveau *et al.* 1956) yields the most active nuclei.

## Introduction

Investigations into the RNA synthesizing reactions of isolated kidney nuclei (Chu and Edelman 1972; Liew *et al.* 1972; Mishra *et al.* 1972) have used the conditions developed by Widnell and Tata (1966) for liver nuclei with little or no modification. This assay involves measuring the RNA synthesizing capacity of nuclei at high and low salt concentrations in the presence of $Mn^{2+}$ or $Mg^{2+}$ ions respectively; the former and latter conditions are taken as a measure of RNA nucleotidyltransferase (EC 2.7.7.6) I and II activity respectively. It has been assumed that the limiting factor in both reactions is the amount of RNA nucleotidyltransferase present, rather than the availability of template. On the basis of these results the above workers have concluded that aldosterone acts to modify the amount of RNA nucleotidyltransferase present in kidney nuclei. Since this assay was developed for liver nuclei and does not appear to have been characterized for kidney nuclei previously, it was felt that the assay should be re-investigated for kidney nuclei. The effect of salt and divalent ions on the reaction was determined and the work was extended by examining the temperature dependence, substrate affinity and effect of exogenous template on the reaction. Our results show that some of the assumptions made are not valid; the results also throw doubt on the conclusions drawn regarding the effect of aldosterone.

## Materials and Methods

*Animals*

Male hooded Wistar rats weighing 140–180 g were fed on laboratory pellets and water *ad libitum*, in a 12-h day, 12-h night environment.

*Chemicals*

All chemicals were A.R. grade. Poly (dA-dT) was purchased from Miles Laboratories, actinomycin D from Calbiochem, nucleotides from Koch-Light Laboratories. α-Amanitin was a generous gift from Professor Th. Wieland, Heidelberg, and calf thymus DNA was a gift from Dr L. A. Burgoyne. Polyethyleneimine (PEI)-cellulose thin-layer plates were from Merck.

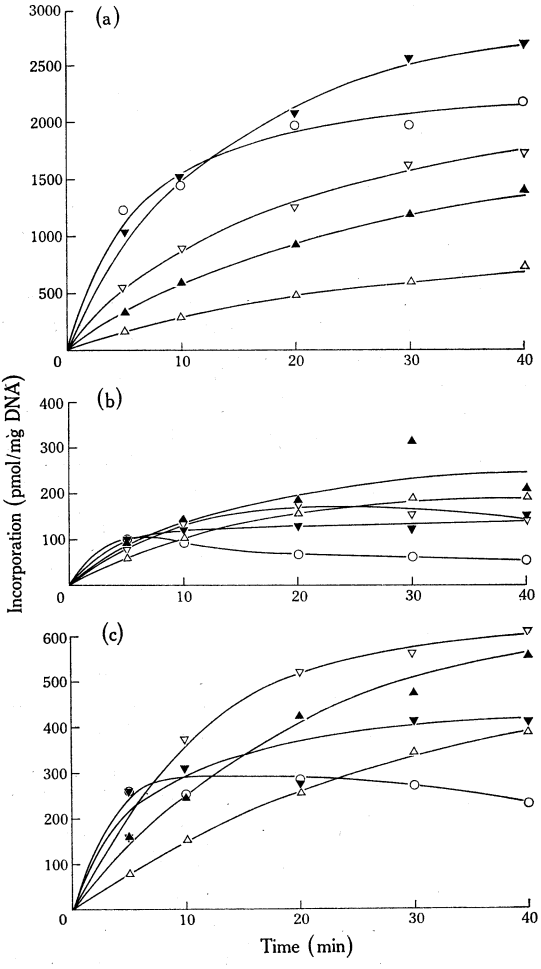


**Fig. 1.** Time course of incorporation of [α-$^{32}$P]UTP into RNA at various temperatures. (*a*) High salt concentration. (*b*) Low salt concentration. (*c*) Low salt concentration with added DNA. Freshly prepared nuclei were incubated for various times under standard assay conditions at the temperatures shown. The reaction was terminated by the addition of 5 ml stopping solution and incorporation into acid-insoluble material determined. ○ 37°C. ▼ 31°C. ▽ 25°C. ▲ 19°C. △ 13°C.

*Isotopically Labelled Compounds*

Uridine-[5-$^{3}$H]-5′-triphosphate (1 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, and [$^{32}$P]$PO_4^{3-}$ from the Australian Atomic Energy Commission, Lucas Heights. [α-$^{32}$P]UTP was synthesized by a modification of the method of Symons (1969) in which the final purification step was by chromatography on Dowex AG 1 X 2 chloride form rather than paper chromatography. [γ-$^{32}$P]GTP was prepared by the method of Glynn and Chappell (1964).

*Preparation of Nuclei and RNA Nucleotidyltransferase Assay*

Unless otherwise stated, nuclei were prepared by the method of Chauveau *et al.* (1956) except that the sucrose was made up in TKM.*

The standard assay system contained (in a final volume of 0·5 ml): 0·1M tris–HCl buffer (pH 8), 1·2 mM ATP, GTP and CTP, 0·04 mM [α-$^{32}$P]UTP (1–2 μCi), 5 mM dithiothreitol, 4 mM $MnCl_2$ or

* TKM = 50 mM tris–HCl (pH 7·5) ,25 mM KCl, 5 mM $MgCl_2$.

5 mM $MgCl_2$, 0·25M, 0·05M or no $(NH_4)_2SO_4$ (referred to as high, intermediate or low salt concentrations respectively), 50 μg heat-denatured calf thymus DNA (where indicated), 2 μg α-amanitin (where indicated) and 0·1 ml freshly prepared nuclei (50–100 μg DNA) resuspended in 50 mM tris–HCl (pH 7·5), 1 mM $MgCl_2$ and 25% (v/v) glycerol. Assay tubes with reaction mixture were preincubated for 4 min and the reaction started by the addition of the nuclei. After 10 min the reaction was stopped by the addition of 5 ml of cold stopping solution. This contained 5% trichloroacetic acid, 1% phosphoric acid, 1% $Na_2SO_4$ and 0·05% AMP. The precipitate was washed twice by centrifugation at 4°C using 5 ml of stopping solution each time, collected on Whatman GF/C glass fibre discs, washed with a further 20 ml of cold stopping solution, 10 ml of 5% trichloroacetic acid–1% phosphoric acid and finally 10 ml of ethanol–ether (3 : 1 v/v). The discs were dried and counted in toluene scintillation fluid [4 g/l 2,5-diphenyloxazole, 0·08 g/l *p*-bis(*O*-methylstyryl)benzene] in a Nuclear Chicago Mark I scintillation counter. Using this procedure zero time values of 50–60 cpm were obtained, of which 20–30 cpm were machine background.

DNA was estimated by Burton's (1956) method.

*Determination of Chain Lengths*

Chain lengths were determined as the ratio of [$^3$H]UMP : [$^3$H]uridine on PEI-cellulose plates following hydrolysis of RNA synthesized from [$^3$H]UTP as descibed by Cox *et al.* (1973). The assay differed from the standard one in that 7 μCi of [$^3$H]UTP was substituted for [α-$^{32}$P]UTP. Counting was performed in Triton X-114/xylene-based scintillation fluid at 25% efficiency (Anderson and McClure 1973).

*Chain Initiation*

The incorporation of [γ-$^{32}$P]GTP into RNA has been used as a measure of initiation (Maitra and Hurwitz 1965). This technique was used in the procedure described below. The standard RNA nucleotidyltransferase assay mixture was modified to contain 40 μCi [γ-$^{32}$P]GTP (0·04 mM) and 1·2 mM non-radioactive UTP. The final precipitate was collected on glass fibre filters and then hydrolysed using 0·5 ml 0·3M KOH at 37°C for 2 h. Hydrolysis was stopped by the addition of 0·5 ml 1M phosphoric acid and the nucleotides eluted from the glass fibre discs with 0·7 ml water. Activated charcoal, 8 mg, was then added to each sample and allowed to stand at 0°C for 90 min. The charcoal was then collected on glass fibre discs, washed with 10 ml 1M phosphoric acid followed by 10 ml 1M phosphoric acid–ethanol (1 : 1 v/v), dried and counted in toluene scintillation fluid.

## Results

*Time Course of the RNA Synthesizing Reaction*

Fig. 1 shows the time courses of [α-$^{32}$P]UTP incorporation into RNA at a variety of temperatures and high and low salt concentrations. The divalent cation throughout is $Mg^{2+}$ (see below). It can be seen that at high salt concentration the incorporation continues at an appreciable rate for 40 min at all temperatures, although in all cases the rate decreases with time and this is more marked at higher temperatures. The addition of exogenous DNA has little effect, suggesting that template availability is not a limiting factor at high salt concentration (C. J. Story and J. F. Wheldrake, unpublished data; Fig. 3). At low salt concentration the situation is somewhat different. In the absence of exogenous template, incorporation continues for a very limited time. If exogenous DNA is added the rate of synthesis is increased at all temperatures and there is a tendency for the reaction to continue for a longer time, as found by Bagshaw and Malt (1971).

If the initial rate of reaction is plotted on an Arrhenius type plot the results shown in Fig. 2 are obtained. From this it can be deduced that the activation energy for the reaction at low salt concentration is less than that for the reaction at high salt concentration. At intermediate salt concentration the activation energy is similar to that at low salt concentration.

*Effect of Varying Salt Concentration*

Fig. 3*a* shows the effect of varying salt concentration when the divalent cation present is $Mn^{2+}$ and Fig. 3*b* shows the results obtained when $Mg^{2+}$ is used. The concentrations of these ions chosen give optimum activity (C. J. Story and J. F. Wheldrake, unpublished data) and are similar to those used by other workers (Widnell and Tata 1966).

It can be seen from Fig. 3*a* that if nuclei alone are present there is a continual rise in activity up to 0·35M $(NH_4)_2SO_4$ and then a decline. If exogenous template is added then the plot is modified by the appearance of a peak of activity at 0·1M $(NH_4)_2SO_4$ and the activity at low salt concentration is markedly enhanced. The addition of α-amanitin at concentrations which suppress kidney RNA nucleotidyltransferase II activity completely (S. D. Deller and J. F. Wheldrake, unpublished data) shows that

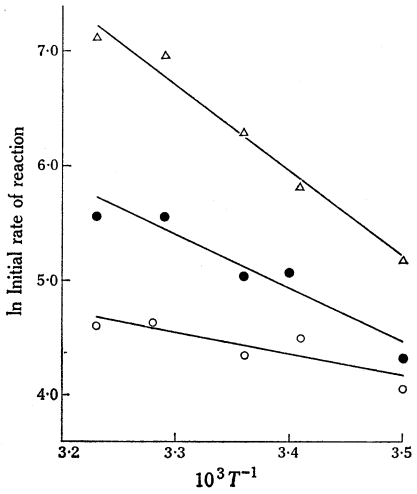


**Fig. 2.** Arrhenius plot of initial rate of reaction. The natural log of initial rate of reaction (obtained by taking the 5-min values in Fig. 1) is plotted against $1/T$, where $T$ is measured in K. △ High salt concentration. ○ Low salt concentration. ● Low salt concentration with added DNA.

RNA nucleotidyltransferase I has an optimum at 0·2M $(NH_4)_2SO_4$ in the absence of added template and at 0·05–0·1M in its presence. From Fig. 3*b* it can be seen that the pattern is similar when $Mg^{2+}$ is the divalent cation. The activity at high salt concentration is very similar but the activity at low salt concentration with added DNA is higher and an increased fraction of it is α-amanitin resistant. Table 1 summarizes the effect of α-amanitin and illustrates the fact that inhibition is less at low salt concentrations (32–40%) than at high salt concentration (86%), in keeping with the view that RNA nucleotidyltransferase I is responsible for the majority of the activity at low salt concentration and RNA nucleotidyltransferase II at high salt concentration (Widnell and Tata 1966; Chu and Edelman 1972). It can also be seen that at low salt concentration exogenous DNA stimulates the α-amanitin sensitive reaction more than the α-amanitin insensitive reaction, i.e. stimulation of RNA nucleotidyltransferase II more than RNA nucleotidyltransferase I.

We also investigated the effect of varying salt concentration at 25°C and obtained similar results. Substitution of native DNA for denatured DNA gave slightly less stimulation and RNA gave no stimulation.

*Determination of the Apparent* $K_m$ *for UTP*

In assaying the activity of the RNA nucleotidyltransferase it is clearly desirable that the nucleotide concentrations used should be well above the apparent $K_m$ value.

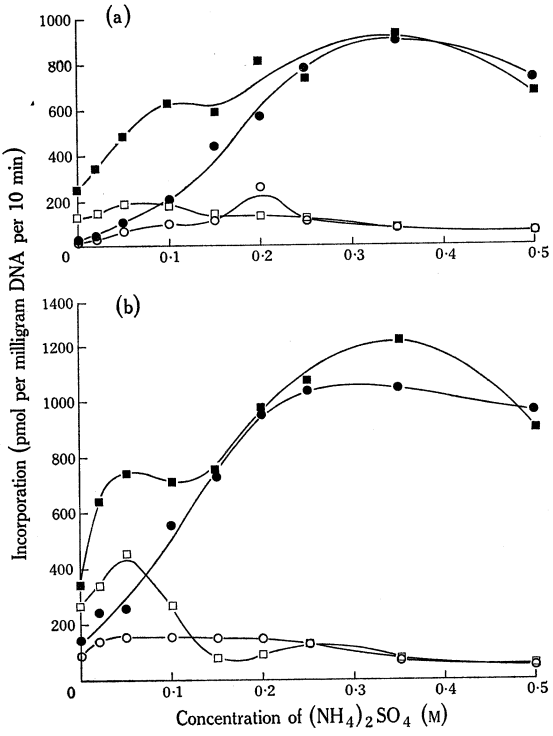


**Fig. 3.** Effect of varying salt concentrations on incorporation of [$\alpha$-$^{32}$P]UTP into RNA. Freshly prepared nuclei were incubated with varying concentrations of added $(NH_4)_2SO_4$ for 10 min. (*a*) 4 mM $Mn^{2+}$ added. (*b*) 5 mM $Mg^{2+}$ added. ● No DNA or $\alpha$-amanitin added. ■ DNA added. ○ $\alpha$-Amanitin added. □ Both DNA and $\alpha$-amanitin added.

Since previous workers have used nucleotide concentrations ranging from 1 $\mu$M or less (Kochakian *et al.* 1971; Trachewsky and Cheah 1971; Chu and Edelman 1972) to 50 $\mu$M or more (Bagshaw and Malt 1971; Mishra *et al.* 1972) it was decided to determine the apparent $K_m$ value for UTP.

**Table 1. Percentage inhibition by α-amanitin of RNA nucleotidyltransferases assayed in the presence of 4mM $MnCl_2$ or 5mM $MgCl_2$**

α-Amanitin was added for each assay. $Mn^{2+}$ values given are averages of eight determinations. Except for intermediate salt conditions (two determinations) $Mg^{2+}$ values given are averages of four determinations

| | Salt concentration of assay | | | | | |
|---|---|---|---|---|---|---|
| | High | High +DNA | Intermediate | Intermediate +DNA | Low | Low +DNA |
| $Mn^{2+}$ | 86·0 | 85·0 | 42·3 | 58·8 | 40·3 | 52·6 |
| $Mg^{2+}$ | 85·8 | 84·7 | 46·7 | 41·9 | 32·1 | 20·3 |

Our results showed that the apparent $K_m$ value at low salt concentration is 8·4 $\mu$M in the absence of $\alpha$-amanitin and 8·7 $\mu$M in its presence. At high salt concentration the corresponding values are 14·4 $\mu$M and 18·9 $\mu$M respectively. The observation that

at low salt concentration $K_m$ is unaffected by α-amanitin suggests that both nucleotidyltransferases have similar affinities for UTP. At high salt concentration the residual RNA nucleotidyltransferase, which is a α-amanitin resistant, appears to have a slightly lower affinity than the α-amanitin-sensitive form which makes up the bulk of the activity. It is clear, however, that the affinity of both forms for UTP is lower at high than at low salt concentration and that the enzymes should be assayed at 40 μM UTP or more.

**Table 2. Variation in RNA synthesizing activity of nuclei isolated by various procedures**

Nuclei were prepared by the method shown and assayed at high, intermediate and low salt concentrations. For details of preparation methods see text and Dounce and Ickowicz (1969). Values are pmol incorporated per milligram DNA per 10 min. Values in parenthesis are percentage activities

| Salt concn of assay | 5 mM $CaCl_2$, 2·2M sucrose | Modified Blobel and Potter method | 0·44M sucrose, pH 5·8 | Triton X-100 | Citric acid | Standard TKM |
|---|---|---|---|---|---|---|
| High | 505 (100) | 273 (100) | 311 (100) | 128 (100) | <1 | 492 (100) |
| High+DNA | 504 (100) | 320 (117) | 385 (123) | 131 (102) | <1 | 513 (104) |
| Intermediate | 199 (39) | 120 (44) | 112 (36) | 39 (31) | <1 | 199 (41) |
| Intermediate +DNA | 411 (81) | 246 (90) | 259 (83) | 67 (52) | <1 | 405 (82) |
| Low | 109 (22) | 45 (17) | 30 (10) | 18 (14) | <1 | 108 (22) |
| Low+DNA | 212 (42) | 95 (35) | 109 (35) | 26 (20) | <1 | 204 (42) |

*Nuclei Preparation Methods*

Since it has been shown that the method of isolation of nuclei has a marked effect on the nuclear protein composition (Dounce and Ickowicz 1969), it was decided to determine the extent to which RNA synthesizing activity was affected. Table 2 shows the results of this series of experiments. It can be seen that the highest activities are obtained when nuclei are isolated in TKM by the Chauveau method (Chauveau *et al.* 1956) or in the presence of $Ca^{2+}$ ions. Other methods give somewhat lower activities and isolation in the presence of detergent or citric acid gives much lower activities. The relative activities under a variety of conditions appear to be remarkably constant, with the possible exception of isolation in the presence of Triton X-100, which seems to decrease the effect of added DNA.

*Characteristics of the RNA Synthesized by Kidney Nuclei*

The apparent chain length was determined as the ratio of UMP : free uridine following alkaline hydrolysis, by assuming that uridine occurs as the terminal nucleoside with the same frequency as uridine incorporation within the chain. In this series of experiments, [$^3$H]UTP was used rather than [α-$^{32}$P]UTP so that this ratio could be determined. The results are shown in the following tabulation:

| | Salt concentration of assay | | | | | |
|---|---|---|---|---|---|---|
| | High | High +DNA | Intermediate | Intermediate +DNA | Low | Low +DNA |
| UMP : uridine ratio | 54·5 | 48·6 | 18·2 | 39·1 | 7·1 | 27·9 |

The values are averages from four experiments, and 4 mM $MnCl_2$ was used. Incorporation ranged from *c.* 30 to 5000 cpm above background.

It is apparent that (1) the chain length increases with increasing salt concentration, (2) the addition of exogenous template results in the synthesis of longer chains, except at high salt concentration where this template is not used (see below), and (3) the chains under low salt conditions are extremely short and might be expected to represent almost exclusively the completion of pre-existing chains. However, this does not appear to be the case (see below).

The rate of initiation of new chains was also measured by following the incorporation of [$\gamma$-$^{32}$P]GTP into RNA. Contrary to expectations there was no decrease in initiation at low salt concentration but rather an increase. The values obtained were 2·9 pmol incorporated per milligram DNA per 10 min at high salt concentration, 5·4 pmol at intermediate salt concentration and 5·7 pmol at low salt concentration. These determinations were all performed without added DNA present. Initiation with added DNA present could not be determined because the large amount of deoxynucleotides present competed with the radioactive nucleotides for binding to the charcoal used in the assay procedure,

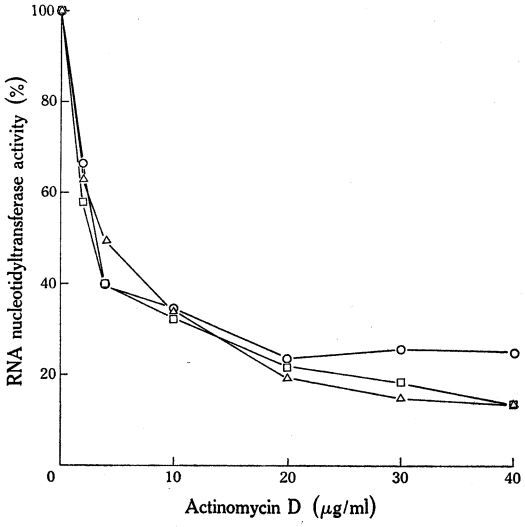


**Fig. 4.** Effect of actinomycin D on RNA nucleotidyltransferase activity of isolated nuclei. Assays were performed under standard conditions with actinomycin D added as shown. The values without added actinomycin D were 43, 204 and 954 pmol per milligram DNA per 10 min. ○, □, △: low, intermediate and high salt concentrations respectively.

*Suppression of the Endogenous Template Activity*

The inhibitory effect of increasing actinomycin D is shown in Fig. 4. Although the final percentage inhibition at low salt concentration is less than that at high salt concentration, this is slightly misleading because the residual activity is much less at low than at high salt concentration.

If the endogenous template is now replaced by poly (dA-dT), which is not susceptible to actinomycin D inhibition, then the results are as shown in the following tabulation:

| | Salt concentration of assay | | | | | |
|---|---|---|---|---|---|---|
| | High | High + poly (dA-dT) | Intermediate | Intermediate + poly (dA-dT) | Low | Low + poly (dA-dT) |
| Activity | 77·2 | 106·0 | 24·4 | 319·0 | 9·0 | 63·0 |

Actinomycin D (30 $\mu$g/ml) was added to the standard assay mixture, together with poly (dA-dT) (0·62 optical density units) where indicated and RNA synthesizing capacity determined. The results show that exogenous template is used extensively

at low and intermediate salt concentrations but only minimally at high salt concentration. The high activity at intermediate salt concentration is consistent with the data shown in Figs 3*a* and 3*b*, which suggests that this represents the salt concentration giving maximum DNA stimulation.

## Discussion

In general it has been assumed that the limiting factor in RNA incorporation studies in whole kidney nuclei is the amount of RNA nucleotidyltransferase (Chu and Edelman 1972; Liew *et al.* 1972; Mishra *et al.* 1972) and the activity at low salt concentration has been equated with ribosomal RNA synthesis, the activity at high salt concentration with messenger RNA synthesis. However, the results described in this paper show that both these assumptions are only partially true. In agreement with Chu and Edelman (1972) we have found that approximately 15% of the activity at high salt concentration is α-amanitin resistant and approximately 60% of the activity at low salt concentration is α-amanitin resistant in the absence of added DNA (Table 1, Figs 3*a*, 3*b*). If α-amanitin-resistant activity is equated with RNA nucleotidyltransferase I activity, then these values give an estimate of the relative activities of RNA nucleotidyltransferases I and II under these conditions. If exogenous DNA is added it stimulates incorporation markedly at low and intermediate salt concentrations. This fact implies that availability of template is limiting at low salt concentration rather than the amount of enzyme.

Contrary to expectations the primary effect of high salt concentration does not seem to be an increase in the rate of initiation but rather an increase in the chain length (see tabulation). The ratio of 3′ terminal uridine to uridine within the chain increases 7–8 times as added ammonium sulphate decreases from 0·25M to zero while the initiation rate does not decrease but rather tends to increase. An alternative method of calculating chain lengths is to calculate the ratio of incorporation at the 5′ end (measured as [$\gamma$-$^{32}$P]GTP incorporation) to the intrachain incorporation (measured as [$\alpha$-$^{32}$P]UTP incorporation). When this was done the values in Table 3 were obtained.

**Table 3. Ratio of [$\alpha$-$^{32}$P]UTP incorporated to [$\gamma$-$^{32}$P]GTP incorporated and estimates of chain length**

| Salt concn of assay | Ratio [$\alpha$-$^{32}$P]UTP to [$\gamma$-$^{32}$P]GTP | 'Corrected' chain length | Ratio [$\gamma$-$^{32}$P]GTP lengths to [$^{3}$H]UMP lengths |
|---|---|---|---|
| High | 208·80 | 417·6 | 7·66 |
| Intermediate | 38·70 | 77·4 | 4·26 |
| Low | 7·05 | 14·1 | 1·98 |

This method will be incorrect for two reasons: (1) Because GTP is used as the initiation nucleotide, whereas UTP is used for all other experiments, the data will be inaccurate to the extent that UTP incorporation is not equivalent to GTP incorporation. However, this effect is probably relatively minor. More importantly, initiation is not random and this method of estimating chain lengths assumes this to be the case. Since initiation is primarily from ATP or GTP (Chambon *et al.* 1970), the crude values in Table 8 should be approximately doubled. (2) This technique assumes that all chains are synthesized *de novo*. This assumption will obviously be invalid but a comparison of the results obtained by this technique and those obtained by the alternative method

(Table 3) yields useful information. It is suggested that many of the chains synthesized at low salt concentration are short and synthesized *de novo* whereas those synthesized at high salt concentration are primarily the completion of pre-existing chains and are much longer. This suggests that the proteins attached to chromatin are not restricting initiation so much as preventing the continued synthesis of chains shortly after initiation. The kinetics of incorporation at low salt concentration (Fig. 1) are compatible with this view.

Although the activity of whole nuclei is highest at high salt concentration [0·25M $(NH_4)_2SO_4$], it has been shown that isolated RNA nucleotidyltransferases have salt optima well below that concentration (Roeder and Rutter 1969). The fact that the affinity of the nucleotidyltransferases for UTP is decreased and the utilization of added DNA totally prevented (Fig. 3) supports the view that this is also the case in whole nuclei and that the increased activity is caused by the removal of inhibitory proteins at high salt concentration. This is reinforced by the fact that if endogenous template activity is suppressed by the addition of saturating amounts of actinomycin D, then exogenous template [poly(dA-dT)] is much more efficiently used at intermediate salt concentration [0·1M $(NH_4)_2SO_4$] than at high salt concentration [0·25M $(NH_4)_2SO_4$].

The low activity of nuclei isolated by a citric acid procedure is not surprising in view of the low pH involved, and the deleterious effect of triton X-100 probably reflects leakage of the enzyme from the nuclei during isolation. In their paper, Dounce and Ickowicz (1969) recommended the 0·44M sucrose, pH 5·8, method for the isolation of nuclei when studies on nuclear proteins are to be undertaken. In our experiments we found no advantages in this method and, in fact, the enzyme activities obtained are somewhat lower than other methods. However, it should be pointed out that if inhibitory proteins (such as histones) are lost preferentially then the RNA synthesizing capacity might be raised and thus our results are not incompatible with their data.

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