THE EFFECT OF DEOXYRIBONUCLEASE ON PRECURSOR INCORPORATION INTO RIBONUCLEIC ACID AND THE NATURE OF A DIPHENYLAMINE - REACTIVE CHROMOGEN.

By

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ABSTRACT

The effect of incubating isolated rat liver nuclei with deoxyribonuclease on the incorporation of C^{14}-labeled precursors into ribonucleic acid of reconstituted homogenates was studied.

When 66-76% of deoxyribonucleic acid was removed from the nuclei by deoxyribonuclease, only a 12-18% and 38-40% decrease in the specific activities of nuclear and cytoplasmic ribonucleic acid was observed. This result indicates that deoxyribonucleic acid does not play a major role in precursor incorporation into nuclear ribonucleic acid.

Deoxyribonucleic acid gives a blue color with diphenylamine. Destruction of deoxyribonucleic acid by deoxyribonuclease leaves a diphenylamine-reactive chromogen which could be extracted by hot trichloroacetic acid from tissues. Investigation of this chromogen shows that it is probably a nucleic acid-like substance tightly bound to proteins and that it cannot be extracted by procedures which usually extract nucleic acids from tissues. Pyrimidine and purine bases and phosphate are present in the diphenylamine chromogen while deoxyribose is not present since the chromogen gave no color in the reaction specific for deoxyribose.

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I N T R O D U C T I O N

DNA* as Genetic Material:

Since nucleic acids are thought to play important roles in biochemical processes of the body, they have long been the subject of intensive investigation. Early workers studied the nature of genes and substances that might constitute the genetic material in the body. Morgan and his co-workers (1, 2) were among the first to show the close relationship between the chromosome movements and the movements of the genetic determinants deduced from the Mendelian genetics. This fact was enough to consider the chromosomes as a "gene carrier".

The next stage of this work was to look for the substance responsible for genetic activity in the chromosomes. In 1922, Feulgen and Rossenbeck (3) discovered a color reaction of DNA which they used to prove that DNA is localized in the nuclear chromatin. Mirsky and Pollister (4) also showed that the protein extractable from tissues with strong salt solutions is DNA-protein and that this extraction causes a loss of stainability of nuclear material. The transformations of type specific pneumococcal bacteria were the first direct evidence that DNA might act as a carrier of genetic information (5).

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* Abbreviations: DNA, deoxyribonucleic acid; DNA-ase, deoxyribonuclease; RNA, ribonucleic acid; RNA-ase, ribonuclease; n-RNA, nuclear ribonucleic acid; c-RNA, cytoplasmic ribonucleic acid; TCA, trichloroacetic acid; PCA, perchloric acid; DPA, diphenylamine; O.D., optical density.

When other constituents such as histone and other proteins (6, 7, 8, 9) were found in the chromosomes, it was no larger possible to claim that DNA was the only compound playing a genetic role. However, Felix (10) and Mirsky et al. (7) have reported metabolic studies indicating these non-DNA components of chromosomes probably do not have genetic activity. Quantitative cytochemical studies also point definitely towards DNA as the carrier of the genetic information. For example, it was shown that DNA is constant in the (diploid) somatic nuclei of various tissues within one species and that haploid sperm cells have half the amount of the diploid cells (11, 12). Mirsky and Ris (11) showed that the nuclear proteins do not exhibit this constancy or the haploid to diploid ratio, expected of genetic material.

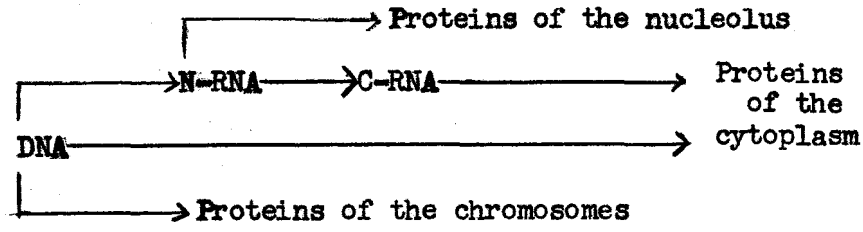
Brachet (9) in his discussion of the role of DNA in heredity and protein synthesis, suggests seven different lines of evidence for the genetic role of DNA: 1) the DNA from each species has a ratio of bases that is characteristic of the species; 2) the specific localization of DNA in chromosomes; 3) the approximate constancy of DNA per chromosome set; 4) the relative metabolic stability of DNA; 5) the effects of mutagenic agents on DNA; 6) the role of DNA in phage reproduction, and finally, 7) the identity of the bacterial transforming agents with DNA

Relation of DNA to Protein Synthesis:

Since genes control the reactions that occur in the body, the genes must simultaneously control the synthesis of enzymes that carry out these reactions. Caspersson (13, 14) originally suggested a direct relation between genes and protein synthesis and he attempted to prove

that the nucleus is the site of protein synthesis. Allfrey (15, 16) and Mirsky (17) studied the effect of DNA on the synthesis of proteins in isolated nuclei and successfully demonstrated that DNA removal inhibits incorporation of a radioactive precursors into nuclear proteins. Gale (18) Brachet and Chantrenne (19) and Mirsky et al. (7) confirmed these findings. These observations do not mean, however, that all protein synthesis is DNA dependent. Allfrey (16, 20) believes that DNA is directly involved only in the synthesis of proteins of the genes.

Other investigators have demonstrated that protein synthesis is completely independent of the nucleus in some types of cells (19, 21, 22). These findings suggest that DNA controls protein synthesis only in an indirect way. The theory advanced by a member of workers (discussed in detail later) is that DNA controls RNA synthesis which in turn, controls the synthesis of proteins (23, 24, 25). Thus DNA will indirectly affect protein synthesis through the synthesis and activity of RNA. Brachet (9) has suggested a scheme to explain the relationship between nucleic acids and protein:



Relation of RNA to Protein Synthesis:

The first evidence of the association of RNA with protein synthesis was obtained independently by Caspersson (12) and Brachet (25) by means of cytochemical studies. In 1951, Leslie and Davidson (27) proved by means of quantitative methods that the synthesis of RNA always precedes protein synthesis in an embryo. It was later found that a direct correlation exists between the RNA content of a cell and its ability to synthesize proteins (28, 29). Gale and Folkes (30) and Allfrey et al. (31) showed that amino acids are incorporated into a bacterial ribonucleoprotein complex, this incorporation was very easily inhibited by the addition of ribonuclease. These findings all provided evidence that RNA plays a role in protein synthesis.

A number of workers have concluded that the most active protein synthesis occurs in microsomal fraction because it is the most active in incorporating labeled amino acids into protein molecules. (32-36). The high activity of the microsomes in protein synthesis apparently due to its high RNA content (31, 37) since RNA-ase inhibits the incorporation of animo acids into protein molecules. (31, 38-42). More direct evidence for the role of RNA in protein synthesis was obtained by Webster & Johnson (39) who showed that addition of RNA to RNA-ase treated microsomes restored amino acid uptake.

In addition, it has recently been shown that the essential components required for protein synthesis are microsomal ribonucleoprotein particles, certain enzymes in the soluble protein fraction, ATP, GDP or GTP and a nucleoside triphosphate-generating system (43-45). A mechanism for protein synthesis suggested by Hoagland et al. (46) is that activated amino acids react with S-RNA (a soluble RNA) in the cell

sap and the activated amino acids are subsequently transferred to the microsomes where they polymerize to form protein under the influence of microsomal RNA.

Additional studies of the various phases of the relation between nucleic acids and protein synthesis have employed microbial systems. Cohen and Barner (47) demonstrated that in a thymine-less E. coli, protein synthesis parallels RNA synthesis even if DNA synthesis is suppressed. Ben-Ishai and Volcani (48) showed that the inhibition of RNA synthesis leads to the inhibition of protein synthesis in a thymine-less E. coli, while the reverse is not true. It has also been shown that protein synthesis never occurs without a simultaneous increase in RNA. (49)

Pardee et al.(50, 51) and Spiegelman et al. (52) also investigated this observation and proved that purine and pyrimidine-requiring mutants of E. coli can not synthesize an induced enzyme unless the required bases are present simultaneously. They also showed that inhibition of DNA synthesis does not have any effect on enzyme synthesis whereas a 50% inhibition of RNA synthesis completely stops any further enzyme synthesis. These findings all furnish additional proof that protein synthesis is closely related to the synthesis of new RNA. In fact, Brachet (9) concludes that the synthesis of a new RNA molecule must accompany the synthesis of enzymes.

It appears furthermore, that a specific RNA is formed for each specific enzyme. Kramer and Straub (53) showed that a new type of RNA was synthesized during the induced synthesis of penicillinase in bacteria. They claim that the RNA isolated from strains having penicillinase as a normal constituent can induce the synthesis of this enzyme

even in the absence of penicillin as an inducer. Chantrenne (54, 55) confirmed that the synthesis of a specific enzyme is closely linked with the synthesis of a new, possibly specific RNA. He also showed that new RNA molecules are synthesized when catalase synthesis is stimulated by the presence of oxygen in non respiring yeast cells.

Nuclear Synthesis of RNA:

From the foregoing discussion it is clear that RNA is closely related to the synthesis of protein and that RNA may therefore control protein synthesis. This is in agreement with the previously mentioned theory that DNA controls the synthesis of RNA which, in turn, controls the synthesis of protein. However, it has also been pointed out that the microsomes are the most active protein-synthesizing fraction. Since DNA is located only on the chromosomes in the nucleus, it is necessary to explain how information can be transferred from the genes to the microsomes.

The best hypothesis available at present to explain this information transfer has been advanced by Beadle (56, Gale (24), Dounce (57), and Muller (58). They propose that n-RNA is synthesized under the influence of the genetic material, DNA, in the nucleus, and the n-RNA then diffuses out to the cytoplasm where it controls protein synthesis. Thus, the first problem is to prove that RNA is synthesized in the nucleus.

Studies on intact cell systems have indicated that considerable RNA synthesis occurs in the nucleus. Marshak and Calvet (59) were among the first to demonstrate this fact by injecting P^{32} intravenously into rabbits and studying the rate of incorporation into different fractions of RNA at intervals after the injection. They found that initially

the specific activity of n-RNA was much higher than that of c-RNA. Then the specific activity of n-RNA decreased with time while the specific activity of c-RNA increased until both reached the same level. This finding is in complete agreement with the hypothesis that n-RNA is the precursor of c-RNA.

In a variety of experimental systems, including amoeba, acetabularia and frog oöcytes, investigators confirmed the above finding (60, 61). In addition, Brachet (62) demonstrated that the nucleus is the first site of RNA reappearance after living Amoeba have been treated with RNA-ase. In very recent work, Zalokar (63) proved definitely that tritiated uridine exclusively appeared in the nuclei when Neurospora were exposed to uridine-H^3 for only one minute.

Site of RNA Synthesis in the Nucleus:

Thus it is clear from the above findings that RNA is synthesized in the nucleus, but the location of this synthesis within the nucleus itself is still unsettled. There are three main theories dealing with the site of RNA synthesis in the nuclei themselves.

The first theory was put forward by Ficq (64, 65) through autoradiographic studies on frog oöcytes and starfish. He demonstrated, that the nucleolus rather than the chromosomes is the primary site of incorporation of labeled adenine or orotic acid into RNA. McMaster-Kaye and Taylor (66) also observed by means of autoradiography that, in Drosophila salivary gland cells, RNA appeared first in nucleoli and later in the chromosomes and cytoplasm of the cell. Additional studies on the primary site of n-RNA synthesis in the nucleus are in agreement with the fact that radioactive precursor are first incorporated into the nucleolar

RNA rather than into the chromosomal RNA (67, 68).

This interpretation that RNA is first synthesized in the nucleoli casts some doubt on the hypothesis that RNA carries information from genes to the cytoplasm since the genetic material is localized exclusively in the chromosomes and chromosomal RNA should therefore become radioactive before nucleolar RNA.

A second theory was suggested by Woods et al.(69) to explain this discrepancy. They showed that when seedlings of Vicia faba (foul bean) were grown on a solution containing H^3-cytidine for 1 hr. the radioactive precursor was mainly incorporated into nucleolar RNA and a very little was found in chromosomal RNA. The authors presented a hypothesis that small RNA molecules are synthesized in close proximity to the DNA of the chromosomes and as soon as they are formed they are transported to the nucleolus where they remain for a time, possibly bound to protein. This would explain why higher radioactivity is found in the nucleolus than in the chromosomes even though synthesis occurs on the chromosomes.

Goldstein and Micou (70) studied the primary site of m-RNA synthesis in human amnion cells. They found that when the cells were exposed to a growth medium containing H^3-cytidine for brief periods of 2, 5 and 10 minutes, the radioactive precursor was mainly (72% incorporated into chromosomal RNA during the first 5 minutes. This supports the view that the chromosomes are the primary site of RNA synthesis in the nucleus. However, there was a significant increase in incorporation into nucleolar RNA between 5 and 10 minutes of incubation, indicating that RNA moves from the chromosomes to the nucleolus during this interval.

In other cases, RNA is clearly synthesized on the chromosomes. (71, 72, 63, 73) For example, Brachet and Ficq (74) showed that the incorporation of radioactive adenine into the RNA-containing loops of the lamp brush chromosomes proceeds very quickly in the germinal vesicle of amphibian oöcytes. Thus, although nucleolar RNA may become radioactive before chromosomal RNA, the findings, just discussed support the idea that the RNA is probably first synthesized in the chromosomes and is later transported to the nucleoli in some cases.

A third theory proposes the reverse of the second theory. Perry (75) has demonstrated that in a tissue culture of Hela cells some n-RNA is first synthesized in the nucleolus, is then transferred to the chromosomes and finally is transferred to the cytoplasm. However, he also believes that another n-RNA fraction is synthesized on the chromosomes themselves and that it moves directly out to the cytoplasm. This theory takes into account the known fact that there are two different fractions of nuclear RNA. (76, 77-81)

It is apparent from the preceding discussion that the nucleus is the site of particularly active RNA synthesis. However, it is not at all clear whether the RNA is first synthesized in the chromosomes and is then transferred directly to the cytoplasm or whether the RNA passes through the nucleolus before transfer to the cytoplasm. In fact, as Perry has pointed out, the reverse may even occur, with RNA being synthesized in the nucleolus and then being transferred to the chromosomes before it passes on to the cytoplasm.

There is evidence to support all of these views in at least one biological system. However, it seems likely that some of the results obtained are probably artifacts due to the experimental methods or to

the interpretation, and they may not represent the actual *in vivo* mechanism. The mechanism for gene action is so basic and is of such fundamental importance that it would be expected to be identical in all living organisms.

RNA Synthesis by the Cytoplasm:

As discussed above, the nuclei are definitely active in RNA synthesis, even though the exact intra-nuclear site is not established. It is desirable at this point, then to consider the question of whether or not the cytoplasm is capable of any RNA synthesis. A few workers have shown that the nucleus is the only site of RNA synthesis although their results were questioned by other workers(82). For example, Prescott (83, 84) has found that neither anucleate *Amoeba proteus* or anucleate *Acanthamoeba* could incorporate C^{14}-uracil or C^{14}-orotic acid into RNA. Plant (82) reported that when the same system used by Prescott was tested in their laboratory with the addition of higher concentrations of C^{14}-uracil the enucleated *Amoebae* incorporated the precursor into RNA.

Several workers have studied this particular problem in *Amoebae* proteins and generally agree that the anucleate *Amoeba* is capable of incorporating C^{14}-adenine (85, 86) and C^{14}-uracil (82) into RNA. Brachet and Ssafarz (87) showed that an enucleated *Acetabularia* could incorporate radioactive precursor into cytoplasmic RNA. Brachet *et al.* (88) also have demonstrated in acetabularia that during the first week RNA synthesis in an anucleate half is faster than in the nucleated half. This suprising result may be explained if it is assumed that the nucleus depletes the cytoplasm of the precursors it needs for RNA synthesis. Thus, the removal of the nucleus would results in an increase in the rate of cyto-

plasmic synthesis of RNA. These findings indicating RNA synthesis in the cytoplasm have been confirmed by many workers in a variety of systems (89, 76, 90, 91, 92, 93).

Further evidence for an independent cytoplasmic synthesis of RNA is the difference of purine/pyrimidine ratio between the cytoplasmic and nuclear RNA (94-96, 97).

Many workers (76, 77, 98) report that despite the chemical similarity of nucleotide content of c-RNA and n-RNA, differences in specific activity indicate that these fractions are synthesized independently. Osawa et al. (77) showed that P^{32} is incorporated at different rates into microsomal RNA as compared to the PH 7.1 fraction of n-RNA of rabbit appendix and thymus even though the two fractions of RNA have identical base content. Hotta and Osawa (76) further suggested that the cytoplasm is responsible for all c-RNA synthesis. Barnum et al. (98) agree with this suggestion and they point out that no conclusive experiments exist which definitely prove that n-RNA is transferred to the cytoplasm.

Theoretical Basis for the Transfer of RNA from the Nucleus to the Cytoplasm:

As mentioned before many investigators have concluded that the nature of each specific enzyme formed in a given organism is determined by the genes present in the nucleus (99, 56). This phenomenon is best expressed as the well known "one gene - one enzyme" theory of Horowitz, (100) which has been fully discussed by Lederberg (101) and Yanofsky (102).

However, this theory does not specify the manner in which genetic information is transferred from DNA to the enzymes. This information can not be transferred directly to the cytoplasmic enzymes since it has

already been mentioned that DNA is localized exclusively in the nucleus (103, 104). Some workers have claimed the existence of DNA in the cytoplasm of some plants (105) and some eggs (106); but Hotchkiss (107) has pointed out that it is as unjustified to consider all types of DNA to be genetic material as to assume all proteins to be enzymes.

If it is true that DNA found in the nucleus is the genetic material it is necessary to postulate an information transferring agent. Goldstein (108), Brachet (9) and Dounce (109) have suggested that nuclear RNA is synthesized under the control of DNA in the nucleus. The m-RNA is then released to the cytoplasm, where it controls the synthesis of proteins by using the "genetic information" it has "obtained" from the DNA. Beadle (56) has stated specifically that the "genes of cellular forms may function by a process in which genetic information is transferred from DNA to RNA segments, which then serve as templates for construction of macromolecules such as proteins".

These hypotheses suggest that the specificity of an enzyme is indirectly referable to the genes. If DNA is used as a template for the synthesis of RNA, which in turn is used as a template for protein synthesis, it would be expected that every mutational modification in a DNA molecule should produce a corresponding alteration in both RNA and protein (56). Demonstration of such small structural alterations has so far been possible only for a few proteins. Recently, eleven quantitatively different human hemoglobins, with only very slight differences have been isolated (110). It will probably be very difficult, if not impossible, to demonstrate similar slight differences in the RNA or DNA templates used for synthesizing these hemoglobins.

Experimental Basis for the Transfer of n-RNA to the Cytoplasm.

Many types of experimental data have been interpreted as favoring the hypothesis of the transfer of n-RNA to the cytoplasm. The high activity of n-RNA was the first indication which led many workers to suggest that n-RNA could be a precursor of cytoplasmic RNA (13, 14, 9, 60, 111).

James (112) has shown that enucleated halves of Amoebae proteus lose RNA at a more rapid rate than do nucleated halves. This could be interpreted as evidence for a nuclear contribution to the c-RNA. Brachet (113) also showed that the amount of c-RNA in Amoebae proteus decreases markedly in an enucleated cell. However, he feels that this is not enough evidence to prove the existence of transfer of n-RNA to the cytoplasm since c-RNA could disappear rapidly from enucleated cells due to causes other than absence of synthesis of n-RNA.

One of the best demonstrations of transfer of the nuclear RNA to the cytoplasm is the experiment of Goldstein and Plaut (89). They transferred nuclei whose RNA was labeled with P^{32} into unlabeled Amoebae. It was observed that the labeled RNA moved out of the donor nucleus into the host cytoplasm. The nature of the labeled material leaving the donor nucleus is still unknown. However, it is probably not phosphate or mononucleotides because these would presumably have been reincorporated into the RNA of the host nuclei as well as the host cytoplasm. However, no labeling of host nuclei was observed, indicating that the labeled material leaving the donor nuclei was probably polynucleotide in nature, if not unmodified RNA itself. An additional proof that phosphate does not leave the donor nuclei is the fact that after transfer is completed, all the radioactivity of the cell can be removed by treatment with RNA-ase. If phosphate had been transferred, it would have also labeled phospho-

proteins which would not be removed by incubation with RNA-ase.

Goldstein and Micou (67) further showed that in human amnion cells, all labeled RNA moves from the nucleus to the cytoplasm within 24 hours as a complex polynucleotide structure which they believe to be only slightly altered on passage to the cytoplasm.

Schneider (114) showed that radioactive RNA is released to the cytoplasm when isolated rat liver nuclei are incubated with an unlabled cytoplasmic fraction *in vitro*. Logan and Smellie (115) have also found that, in a rat liver homogenate, transfer of RNA from the cytoplasm to the nucleus is a possibility.

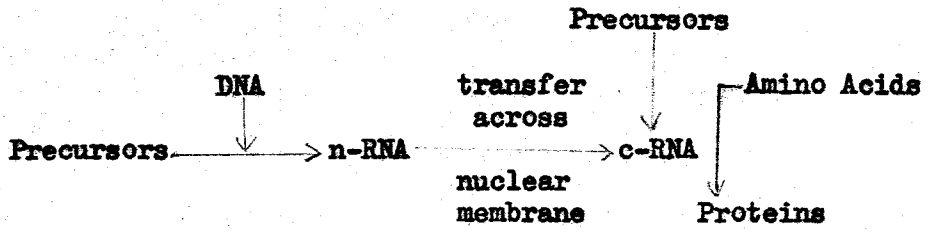
However, several workers (116, 76) do not accept the concept of transfer of n-RNA to the cytoplasm since they feel that the evidence for this transfer is not conclusive. Kay *et al.*(116) suggested that the existence of n-RNA in the cytoplasm may only be due to the breaking of some nuclei during homogenization. In addition, nuclear RNA may leak out of the nuclei during homogenization and may be absorbed on some fraction which it is normally not associated with in the living organism.

In view of the experimental data discussed above it appears evident that in *Amoebae* and other systems such as human amion cells and liver homogenates there is a definite transfer of RNA from the nucleus to the cytoplasm. However, clear cut proof of such transfer in higher animals *in vivo* has not yet been published. Thus, the relation between the n-RNA and c-RNA *in vivo* is still not clear.

Summary:

It is desirable at this point to consider the scheme of events in the transfer of genetic information from the nucleus to the cytoplasm and to point out which steps are established and which steps require

further investigation:



The role of DNA in the biosynthesis of n-RNA is far from clear since in most cases, no DNA requirement can be shown for this step. This question is considered in detail in the next section.

Nuclear synthesis of RNA can be demonstrated in almost all systems studied. However, the exact intra-nuclear site of n-RNA synthesis is not exactly known. Three theories suggested were:

1. n-RNA is synthesized in the nucleolus
2. n-RNA is synthesized on the chromosomes and rapidly transported.
3. n-RNA is synthesized in the nucleolus and on the chromosomes independently. After synthesis, nucleolar RNA is transported to the chromosomes.

From the data presented, it is evident that some n-RNA is transferred to the cytoplasm in Amoeba proteus. In addition, results were discussed above which clearly showed that RNA is synthesized independently in the cytoplasm even if the transfer of n-RNA from the nucleus to the cytoplasm does occur.

The relation between protein synthesis and RNA is well established since RNA is necessary for the synthesis of proteins. It is also fairly well established that for every protein a specific RNA exists in the cytoplasm.

Thus, the whole scheme shown above is far from proved, particular-

ly the role of DNA in RNA synthesis and the transfer of m-RNA from the nucleus to the cytoplasm require considerable further investigation. The experiments described in this thesis were undertaken to extend knowledge of the role of the DNA in the synthesis of nuclear RNA.

ADDITIONAL RECENT FINDINGS

Krakow and Kammen (195) showed that one of the fractions isolated from disrupted nuclei of calf thymus is capable of incorporating ribonucleotides and deoxyribonucleotides into non-dialyzable acid insoluble material. RNA-ase only slightly inhibited the incorporation of both nucleotides while pancreatic DNA-ase strongly inhibited the incorporation of these nucleotides. Treatment of the resulting material with micrococcal DNA-ase and spleen diesterase indicated that large percentage of the incorporation of radioactive precursors into nuclear fraction is terminal.

Hurwitz et al.(196) have separated an enzyme fraction from E. Coli cells which is capable of incorporating radioactive UMP into polyribonucleotides. The incorporation requires the presence of the four nucleoside triphosphates (GTP, ATP, CTP and UTP) all of which were incorporated into polyribonucleotides which were found to have the properties of RNA. In addition, the incorporation requires the presence of DNA which could not be replaced by RNA since no radioactivity was detected in RNA when DNA was replaced by E. Coli RNA in the system. DNA-ase and RNA-ase treatment of the suspension mixture strongly inhibit the incorporation of C^{14}-UMP into RNA.

In an independent work Stevens (197) showed that in an enzyme system of cell fractions of E. Coli. extracts adenine ribonucleotide from radioactive ATP is incorporated into an RNA fraction which is similar to insedimentation properties to the small ribonucleoprotein particles. This enzyme system requires the presence of GTP, UTP and CTP. When DNA-ase and RNA-ase were added to the system the incorporation into RNA fraction was inhibited by 100%. Stevens also demonstrated that the incorporation of AMP is more than 90% into internucleotide linkage in the RNA fraction.

Similarly, Weiss (198) have demonstrated that in an enzyme system of rat liver, cytidine P^{32}-P-P is incorporated into a material which was identified as RNA and that the incorporation requires the presence of all four triphosphates. He also showed that incorporation has taken place throughout the entire polynucleotide chain rather than at the end of the chain. Preincubation of the enzyme system with low concentrations of DNA-ase completely inactivates the system.

However, the way DNA affects the incorporation of radioactive precursors into RNA in the systems discussed above was not clear to the workers themselves who demonstrated this effect.

Recently, Furth et al. (199) after succeeding in purifying the enzyme responsible for RNA synthesis from E. Coli proved that RNA synthesis is completely dependent on the addition of DNA to the system. They also showed that RNA synthesis and the different requirements for this synthesis depend on the nature of DNA added as an inducer. For example, the four nucleoside triphosphates are required and all are incorporated into the product when thymus DNA is added. While only ATP is required and it is the only nucleoside triphosphate that is utilized for RNA synthesis when Poly T is added. Furthermore, the enzyme system does not need any RNA "primer" and the addition of RNA-ase does not affect AMP incorporation when Poly T is used as an inducer while DNA-ase addition does inhibit the incorporation.

It may be seen from the above discussion that DNA plays an important role in RNA synthesis of an enzyme system isolated from either E. Coli or rat liver homogenate However, it is not yet well understood whether DNA is the only specific substance that controls RNA synthesis or whether any DNA-like substance might control RNA synthesis.

PART II

EFFECT OF DEOXYRIBONUCLEASE ON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO RIBONUCLEIC ACID

EFFECT OF DNA-ASE ON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO RIBONUCLEIC ACID

It has been proposed that RNA carries genetic specificity from DNA of the chromosomal gene to sites of protein synthesis in the cytoplasm (56, 24, 109, 58).

The requirement of RNA for protein synthesis (45, 46, 9) is well established. However, little is yet known of the relation of DNA to RNA synthesis. It is possible to study this relation by gradually removing DNA from the nucleus with DNA-ase and determining the change in the rate of incorporation of a radioactive precursor (adenine or orotic acid) into nuclear and cytoplasmic RNA.

Although several authors have made similar studies (117, 118, 119, 120, 20, 121) (See discussion section) no one has attempted to accurately correlate the extent of DNA removal with the rate of incorporation of precursors into RNA. Similarly, no major studies have been made of the effect of DNA-ase on the incorporation of precursors into specific fractions of RNA. This investigation was designed to study these matters in considerable detail.

EXPERIMENTAL

Materials: Rats were supplied by Tierzuchterei Brunger (Halle, Westfalen, Germany). The non-crystalline lyophiled and 1 x-crystalline DNA-ase and highly polymerized DNA were supplied by Worthington Biochemical Corporation (Freehold, New Jersey). Adenine sulphate-8-C^{14} Hemihydrate (specific activity: 26.2 microcuries/mg.) was supplied by the Radiochemical Centre (Amersham, England). Orotic acid-6-C^{14} hydrate (specific activity: 1.15mc/uM) was supplied by Tracer lab.

Incubation of Homogenates and Isolation of Nucleic Acids: Adult female rats were sacrificed and a 20% homogenate of the excised liver in 0.25 M sucrose was prepared. Aliquots of the homogenate were transferred to 50 ml.-erlenmyer flasks containing an equal volume of the following solution: succinate (0.006 M) pyruvate (0.02 M), glutamate (0.02 M), phosphate buffer, PH 7.4 (0.02 M), magnesium chloride (0.006 M), niacin (0.01 M), ATP (0.0008 M), fructose (0.012 M), orotic acid-6-C^{14} (0.5 microcuries/ml.) or adenine-8-C^{14} (1.2 microcuries/ml.), and sucrose (0.25 M). Different concentrations of DNA-ase were added to each flask depending on the experiment. The mixture was incubated by shaking in a water bath at 30° C for 45 minutes and nucleic acids were isolated using the procedure of Schneider (114), which is briefly outlined below.

After incubation, the flasks were chilled in ice and the incubation mixtures were transferred to plastic tubes, rehomogenized with a plastic pestle to break up clumps formed during incubation, and then centrifuged at 600 x g. for 10 minutes in a refrigerated centrifuge to sediment the nuclei. The nuclear pellet was resuspended in 0.25 M sucrose by homogenization and was underlayered with 0.34 M sucrose (122, 123). The resuspension was again centrifuged at 600 x g. for 10 minutes and the supernatant was combined with the first cytoplasmic fraction. The

cytoplasmic and nuclear fractions were precipitated at 0° with enough perchloric acid (PCA) to give a final concentration of 0.3 N PCA, and the precipitate was washed twice with 0.3 N PCA at 0° C. The precipitate was suspended in 3 ml. of 10% NaCl and the pH was adjusted to 7.2 (phenol red). The NaCl suspensions were heated at 100° C for 20 minutes to extract nucleic acids as the sodium salt and the sodium nucleates were precipitated from the extract by the addition of 95% ethanol. The resulting precipitate was sedimented by centrifugation and then resuspended in a 3:1 alcohol:ether mixture, heated at about 50° C for 15 minutes to extract lipids, and centrifuged. Excess alcohol was removed from the tube and the precipitate by brief heating in a water bath at 100° C (Drying was avoided). The pellet was then dissolved in 1 ml. of 0.1 N NH_4OH and aliquots were used for measuring the radioactivity and RNA content. RNA content was determined by the orcinol method (124) with arabinose as a standard.

Incubation of Isolated Nuclei with DNA-ase: After preparing a 20% rat liver homogenate at 0°, 6 ml. aliquots were transferred into test tubes and centrifuged at 600 x g. for 10 minutes at 0° C. The cytoplasmic fractions were kept in centrifuge tubes in the cold and each nuclear pellet was resuspended in 1 ml. of an incubation mixture containing 0.04 M magnesium chloride, 0.02 M phosphate buffer (PH 7), and DNA-ase of various concentrations depending on the experiment. The nuclei plus cofactors and DNA-ase were incubated in a water bath at 30° C for 20 minutes, centrifuged at 600 x g. for 10 minutes, and the supernatants were discarded. The nuclei were then recombined with the corresponding cytoplasmic fractions and 2.5 ml. aliquots of these reconstituted homogenates were used for the standard incubation procedure described above.

Determination of DNA Content: After incubation of the nuclei with DNA-ase, 2.5 ml. aliquots of the reconstituted homogenate were transferred to glass tubes and centrifuged at 600 x g. for 10 minutes to reisolate the nuclei. The supernatant was discarded. The nuclear pellet was precipitated with

ice cold 5% TCA, washed twice with 5% TCA at 0° C, and centrifuged at highest speed to remove most of the TCA from the tissues. DNA was then extracted with 1.3 ml. 5% TCA at 90° C for 15 minutes, centrifuged, and the supernatants were collected. DNA content was determined by a modified Dische reaction (124) using highly polymerized DNA as a standard.

RESULTS

In order to examine the effect of DNA-ase on the incorporation or orotic acid-6-C^{14} into nuclear and cytoplasmic RNA, non-crystalline DNA-ase was incubated with whole rat liver homogenates using the standard incubation procedure (see text). It may be seen from Figure I that although fairly high concentrations of DNA-ase were used, the radioactivity of RNA was not significantly decreased. It seemed unlikely that absence of inhibition was due to low DNA-ase concentration since other workers have used lower DNA-ase concentrations to remove most of DNA (121). Spiegelman (125) believes that DNA-ase may not easily diffuse through the nuclear membrane of the cells and this may be a serious hinderance to the activity of DNA-ase.

In an attempt to overcome this difficulty, rat liver homogenates were preincubated with DNA-ase at 0° C for 3 hours prior to incubation with radioactive precursors. It was thought that preincubation might give the enzyme time to diffuse through the nuclear membrane without affecting the activity of the cells themselves. However, in Figure II, it is clear that the specific activities of n-RNA and c-RNA were found to be almost unaffected by high DNA-ase concentrations even though whole homogenates were preincubated with DNA-ase at 0° C for 3 hours before addition of radioactive precursor.

Since preincubation with DNA-ase at 0° failed to inhibit the incorporation of radioactive orotic acid into RNA, it seemed desirable to try preincubation at higher temperatures. This might possibly permit faster and more complete diffusion of DNA-ase through the nuclear membrane, as well as permitting the enzyme to act on DNA. But, before using the enzyme at higher temperatures, optimum conditions for the incubation had to be determined, since preincubation at elevated temperatures is known to be detrimental to the incorporation of precursors into cells (126).

In an experiment designed to test the new conditions, homogenates were preincubated at 30° C for different intervals of time before addition of orotic acid-6-C^{14}. The results are presented in Figure III, where it may be seen that homogenates may not be preincubated at 30° C for more than 30 minutes, since the specific activity of RNA drops suddenly to low values after that time.

In Figure IV, preincubation at 30° for 30 minutes with crude DNA-ase significantly inhibited incorporation of orotic acid-6-C^{14} into RNA. It was hoped that even more removal of DNA and therefore even more inhibition might be obtained by adding $MgCl_2$ during the preincubation, since it is well established that high Mg^{++} is necessary for the maximum action of DNA-ase (127). In addition, it seemed desirable to begin using crystalline DNA-ase at this point.

The first preincubation experiment with crystalline DNA-ase in presence of $MgCl_2$ failed to produce any incorporation of radioactivity into RNA. Since the time and temperature of preincubation were identical to the previous experiment of Figure III, it was felt that the magnesium ions had inhibited the incorporation. It is known from the work of Schneider (126) that magnesium ions do inhibit incorporation of precursors into RNA. It was therefore desirable to determine optimum conditions for the incorporation of orotic acid-6-C^{14} into RNA as a function of preincubation time in the presence of different $MgCl_2$ concentrations. As shown in Figure V, the specific activity reaches a maximum within the first 10 minutes and then decreases until it reaches almost zero at the end of 45 minutes, regardless of whether the $MgCl_2$ concentration is 0.003 M or 0.006 M.

Using a 15 minute preincubation time and the higher magnesium concentration (0.006 M), it was found (see Figure VI) that increasing amounts of crystalline DNA-ase at low concentrations slightly stimulated rather than inhibited in-

corporation. This indicates that the drop in the specific activity of RNA in Figure IV where non-crystalline DNA-ase was employed, was probably due to impurities or to some unknown contaminants in the cofactor and substrate solutions rather than to DNA-ase activity. Sekiguchi and Sibatani (120) also observed that DNA-ase differs from one batch to another in terms of the effect on the incorporation of radioactive precursors.

Since preincubation with crystalline DNA-ase at these low levels had no inhibitory effect on incorporation (Figure VI) it seemed desirable to double the concentration and to prove that DNA was actually being destroyed. In Figure VII, it can be seen that removal of 45% of the DNA by doubling the concentration of crystalline DNA-ase gave no inhibition of incorporation.

After failing to observe any effect of DNA-ase on the specific activities of cytoplasmic and nuclear RNA in whole homogenates, DNA-ase was incubated with isolated nuclei so that higher concentrations of DNA-ase could be used, the DNA-ase could act directly on the nuclei, and the concentration of $MgCl_2$ could be increased to optimum levels for DNA-ase action without inhibiting subsequent incorporation.

It is known that the optimum concentration of $MgCl_2$ for DNA-ase is 0.04 M (127). However, magnesium at this high concentration is known to inhibit the incorporation of radioactive precursors into RNA (126). It was therefore necessary to determine whether incubating nuclei with 0.04 M magnesium would affect the subsequent incorporation of precursors into RNA.

Nuclei isolated from rat liver homogenates were preincubated with $MgCl_2$ for different lengths of time and nuclei were then recombined with cytoplasmic fractions. The reconstituted homogenates were incubated with

orotic acid-6-C^{14} as shown in Figure VIII. It is clear from the results of two separate experiments that after a slight apparent initial lag, magnesium stimulated incorporation, reaching a maximum at 20 minutes. Longer incubations resulted in significant inhibition of incorporation into n-RNA but affected incorporation into c-RNA to a much less extent.

Using the 20 minutes incubation time that appeared to give maximum incorporation, $MgCl_2$ concentration was varied. The results are shown in Figure IX, where it may be seen that maximum incorporation was obtained with 0.04 M $MgCl_2$ for 20 minutes. Above this concentration the specific activity of the n-RNA decreases while the specific activity of the c-RNA remains almost unchanged. These results indicated that optimum incorporation should be obtained if isolated nuclei were preincubated with crystalline DNA-ase in a solution containing 0.04 M $MgCl_2$ at 30°C for 20 minutes. Isolated nuclei were preincubated under these conditions prior to recombination with the corresponding cytoplasmic fractions to form "reconstituted homogenates".

It may be seen from Table I, that preincubation with crystalline DNA-ase at a concentration of 2-3 mg./ml. removed as much "apparent DNA" as was removed by 5 mg./ml. Even at this high concentration, it appeared that 25% of the "apparent DNA" could not be removed. This fact will be discussed in detail later.

It is also seen that in all three experiments, the removal of 67-76% of "apparent DNA" reduced the radioactivity of n-RNA by 12-18% and c-RNA by 38-40%. The percent inhibition of incorporation was not significantly changed by increasing the DNA-ase concentration from 2 to 4 mg./ml. and 2 to 5 mg./ml. in experiments II and III of Table I. However, in experiment I, increasing the crystalline DNA-ase concentration from 2 to 5 mg./ml. caused the percent inhibition of precursor incorporation to increase from

14% to 51% and from 38% to 68% for n-RNA and c-RNA respectively. It is clear that this inhibition in experiment I is not caused by DNA-ase itself since the decrease in radioactivity on the addition of more DNA-ase was not observed in experiments II and III, where a different batch of DNA-ase was used. Instead, some unknown inhibitors of incorporation must have been present in the first batch of crystalline DNA-ase used in experiment I. Sekiguchi and Sibatani (120) have also mentioned that batches of DNA-ase differ distinctly in their effect on the incorporation.

The results of Table I are plotted on Figure X along with additional data obtained in same experiments. The slight inhibition of incorporation into n-RNA obtained by removing all DNA-ase-labile material is hardly apparent in two of the curves (representing experiments II and III of the Table I).

It should be pointed out that the incorporation of adenine-8-C^{14} into n-RNA is slightly stimulated by 0.5 mg./ml. of DNA-ase as shown in Figure X. This stimulation of incorporation by low concentrations of DNA-ase may be due to the release of some RNA as a result of the action of DNA-ase in destroying the integrity of the chromosomes. The released RNA might be more active in incorporating radioactive precursors than the bound RNA.

The amount of RNA in the nuclear and cytoplasmic fractions were determined during the experiments presented in Figure X. It may be seen from Figure XI that the increase in the amount of nuclear RNA is inversely proportional to the amount of DNA removed by DNA-ase. In addition, an increase in the amount of n-RNA is accompanied by a corresponding decrease in the amount of c-RNA, while the total RNA of the two fractions remains nearly equal.

DISCUSSION

Work during the last few years has helped in understanding the possible biochemical role of DNA in nuclear RNA synthesis. The question is, however, far from settled as the following discussion will indicate.

Sekiguchi and Sibatani (119, 120) studied the role of DNA in two separate experiments. When isolated nuclei of rabbit appendix were incubated with P^{32} in sucrose Tyrode medium, P^{32} was incorporated into DNA, RNA and the organic acid-soluble phosphorus fraction (see Table II). They showed that removal of 70% of the DNA-ase almost completely abolished incorporation of P^{32} into RNA.

In addition, Allfrey et al. (20) briefly stated that removal of 60-80% DNA from an unspecified tissue decreased the specific activity of RNA by 93%. In another experiment, Allfrey and Mirsky (121) showed that in isolated nuclei of calf thymus tissue, the removal of at least 60% of the DNA by DNA-ase inhibits incorporation of adenine-8-C^{14} into n-RNA by 60%. They further demonstrated that the addition of DNA, RNA or polyanions (such as polyethylene sulfonates, heparin and chondroitin sulfate), will restore the ability of DNA-ase-treated nuclei to incorporate radioactive precursors into proteins, RNA and ATP. It was possible to replace two - thrids of the DNA by any of the mentioned polyanions without any apparent loss of ability to incorporate radioactive precursors into nucleic acids and proteins (121). Recently, Sekiguchi and Sibatani (120) have confirmed this finding.

The addition of polycations could not restore the activity of DNA-ase-treated nuclei (121, 120). On the contrary, polylysine inhibits the incorporation of amino acids into proteins in normal nuclei.

The effect of polyanions on the synthetic activity of normal cells was independently interpreted by Sekiguchi and Sibatani (120) and Allfrey and Mirsky (121) on the basis of the following theory: An acid-base balance exists in animal chromosomes. It consists of DNA as the acid component and

histones and protamines as basic proteins that neutralize DNA. These basic proteins are believed to play a role in controlling chromosome function by masking the negative charge of DNA which is directly related to the biochemical activity of the chromosomes themselves. Removal of DNA or addition of a polycation disturbs this neutrality balance and upsets synthetic reactions of the nuclei. Allfrey and Mirsky (128) believe that the primary effect of this blockage is inhibition of ATP synthesis by the nucleus and that this defect prevents nucleic acid and protein synthesis.

Sekiguchi and Sibatani (120) suggest that DNA is not required for the synthesis of n-RNA and that genetic information is transmitted from DNA to n-RNA only indirectly through micromolecules other than DNA. This assumption seems unjustified on the basis of the data they have presented. Although, polyanions must be present in these experiments for restoration of the three fundamental activities of these isolated nuclei (ATP, RNA and protein synthesis), it is unlikely that DNA has no control over the sequence of monomers in RNA. Instead, it is reasonable to assume that proteins and RNA made in the absence of DNA are "nonsense molecules" or polymers containing no genetic information.

In fact, Sekiguchi and Sibatani, themselves (120) and Allfrey and Mirsky (121) agree that the restoration of the activity of DNA-ase treated nuclei of animal cells by any non-specific polyanion does not exclude the specific role of DNA in the incorporation of radioactive precursor since only 60-75% of the DNA was removed by DNA-ase under the best conditions. It is therefore possible that this residual, DNA-ase-resistant 'DNA' controls protein and RNA synthesis after DNA-ase treatment.

Thus, it is evident from the work of Sekiguchi and Sibatani (120) and Allfrey and Mirsky (121) that in isolated nuclei, DNA-ase does decrease incorporation of precursors into RNA. A contrary report has also appeared. Weiss and Gladstone (117) found that DNA-ase slightly depresses the incorpor-

ation of cytidine-P^{32}-P-P into RNA in nuclei isolated from a 20% rat liver homogenate.

Using bacterial systems, Spiegelman (125) showed that in lysed protoplasts of B. megatherium, removal of DNA does not abolish the synthesis of DNA, RNA or protein. This finding was further confirmed by Otsuji and Takagi (129) with lysed protoplasts of E. Coli. It is possible to interpret the results obtained in these bacterial cells systems on the basis of the neutrality balance theory which was discussed before. DNA in bacterial cells, unlike animal cells, is complexed with polyamines of relatively low molecular weight (130). When DNA is removed, these low molecular weight amines may be lost from the protoplast without causing any major disturbance in neutrality balance. Therefore, synthesis of n-RNA can continue in bacteria and there is no necessity to add polyanions.

In the present experiments, the effect of DNA-ase on precursor incorporation into RNA was studied in whole homogenates. The results, discussed in the previous section, showed that complete removal of DNA did not inhibit incorporation of radioactive adenine into n-RNA more than 18%. Incorporation into c-RNA was inhibited to the extent of 40%. These levels of inhibition tend to agree with the inhibition (24%) noted by Weiss and Gladstone (117) and fall between the absence of marked inhibition noted by Spiegelman (125), and the large inhibitions noted by Sekiguchi and Sibatani (120) and Allfrey and Mirsky (121), (almost 100% and 60% respectively).

It may be seen from Table I that DNA-ase inhibits the incorporation of adenine-8-C^{14} into cytoplasmic RNA, although it was expected that DNA-ase would specifically affect only the incorporation of precursors into nuclear RNA. In fact, the inhibition of incorporation of adenine-8-C^{14} into cytoplasmic RNA was greater than the inhibition observed for nuclear RNA at any given DNA-ase concentration.

The decrease in the specific activity of c-RNA can be explained if it is assumed that n-RNA is independently synthesized on the nucleoli and the chromosomes (75) and that only the RNA synthesized in one of these fractions is inhibited by DNA removal. If the fraction inhibited by DNA-ase is the same fraction that is normally released to the cytoplasm during incubation, the decrease in the specific activity of c-RNA would be observed.

However, the greater apparent inhibition of precursor incorporation into c-RNA than into n-RNA may also be due to a change in the relative amounts of c-RNA and n-RNA as a function of DNA-ase concentration. As explained below, it is clear from Figure XI that c-RNA becomes associated with the nuclear fraction at higher concentrations of DNA-ase. Schneider (131) demonstrated that in a rat liver homogenate, the supernatant RNA has a higher specific activity than all the other fractions of cytoplasmic RNA. If this fraction is the one that becomes associated with the nucleus it will cause an increase in the specific activity of n-RNA and a decrease in the specific activity of c-RNA. This might explain why the specific activity of c-RNA is so markedly lowered by DNA-ase.

The change in amounts of n-RNA and c-RNA noted in Figure XI may be due to a disturbance in the neutrality balance of the nuclei, since the removal of DNA by DNA-ase sets free the basic proteins, histones and protamines. As mentioned before, Sekiguchi and Sibatani (120) believe that this disturbance of neutrality balance results in the inhibition of ATP synthesis in isolated nuclei. As a result, the lack of ATP prevents incorporation of radioactive precursors into proteins and RNA. In addition, as mentioned before, Allfrey and Mirsky (121) have demonstrated that in isolated nuclei of calf thymus, the addition of polyanions restores the incorporation of precursors into protein, RNA and ATP.

These workers demonstrated a requirement for polyanions in their systems of isolated nuclei before RNA synthesis would occur after DNA-ase treatment.

However, in the present experiments with reconstituted homogenates the incorporation of adenine-8-C^{14} into n-RNA was not significantly inhibited, although 67-76% of DNA was removed by DNA-ase as shown in Figure X. This failure of DNA removal to inhibit incorporation even without the addition of any polyanion can be explained if it is assumed that some polyanionic substance, contributed by the cytoplasm of the "reconstituted homogenate", becomes bound to the basic proteins of the nucleus and neutralizes them. One available polyanion in the "reconstituted homogenates" would be cytoplasmic RNA.

An indication that RNA becomes associated with nuclei after DNA-ase treatment is furnished by Salganik et al. (118) who showed that when RNA is added to DNA-ase-treated nuclei of calf thymus, the nuclear RNA is increased to levels 2.7-4.4 times higher than the initial RNA content of nuclei.

The data shown in Figure XI where the amount of n-RNA increases and the amount of c-RNA decreases after DNA-ase treatment can best be explained in terms of the binding of c-RNA to basic nuclear proteins as described above. If the highly radioactive supernatant fraction of c-RNA is selectively bound to the nuclei, then an increase in the specific activity of n-RNA and a decrease in the specific activity of c-RNA would be observed. Thus, this would explain the apparent lowering of c-RNA specific activity noted when DNA-ase concentrations are increased.

The amount of DNA replaced by RNA was not more than 38% of the DNA removed. However, this low percent replacement might be dur to the fact that other anionic substances in the system may neutralize a part of the basic proteins.

Regardless of whether the c-RNA becomes associated with the nuclear fraction by the mechanism just postulated or not, it is clear from the results of Figure XI that such association does occur. This finding has serious consequences when interpreting the effect of DNA-ase on the incorporation of precursors into RNA. It is not possible, if binding occurs, to determine the relative amount of radioactivity incorporated by the RNA originally present in the nuclei and the amount incorporated by the cytoplasmic RNA which becomes bound to the nuclei. For example, removal of DNA might possibly result in a complete inhibition of precursor incorporation by the RNA originally in the nucleus. All the radioactivity observed as "apparent nuclear RNA" would then be dur to incorporation by cytoplasmic RNA that becomes associated with the nuclear fraction. Thus, it is difficult to prove that DNA is not required for the synthesis of n-RNA on the basis of the data presented in Figure X.

An even stronger reason why the data of Figure X do not prove that DNA is not required for n-RNA synthesis is that incorporation of precursors <u>in vitro</u> does not necessarily represent synthesis.

Heidelberger <u>et al</u> (132) demonstrated that when adenosine-5-P^{32} was incubated with the cytoplasmic fraction isolated from a 0.25 M sucrose homogenate of rat liver, the label appeared in RNA. When this RNA was hydrolyzed with diesterase which is known to give 5-mononucleotides on cleavage, the radioactivity was found almost entirely in adenosine-5'-phosphate. In addition, when the same RNA sample was hydrolyzed with alkali, the radioactivity was found mainly in 2'- and 3'-cytidylic acid. From these data they have concluded that 5'-mononucleotides are incorporated into RNA without the loss of their phosphorus and adenosine is almost always adjacent to cytidine in the case of the RNA formed by the specific system used.

Canellakis (133) also showed that when doubly labeled adenosine triphosphate (Adenine-8-C^{14}-ribose-P^{32}-P-P) is incubated with a mammalian enzyme system (a dialyzed extract of an acetone powder prepared from the soluble cytoplasmic fraction of rat liver) and when the resulting RNA was hydrolyzed in alkali the radioactive phosphorus was found in 2'- or 3'-cytidylic acid and the labeled carbon in adenosine-8-C^{14}. Canellakis concluded that in mammalian systems, AMP is only incorporated into RNA or "RNA-like material" through terminal attachment by an internucleotide linkage to a cytidylic acid unit on the end of the nucleotide chain. Furthermore, the enzyme system involved in this reaction is specific for the attachment of AMP exclusively to a terminal cytidylic acid of RNA. Zamecnik et al (134) also studied the incorporation of AMP^{32} into RNA of whole rat liver homogenate and their results were in agreement with the work of Canellakis (133).

However, other workers (135, 136) demonstrated that when RNA is labeled with orotic acid-6-C^{14} using a rat liver cytoplasmic fraction, the radioactivity after alkaline hydrolysis was found entirely in entirely in 2'- and 3'-uridine-P. This indicates that orotic acid is incorporated into the inner part of the chain since uridine would be the main product if orotic acid was incorporated only into the terminal position of RNA molecule.

It is probable that the incorporation of adenine-8-C^{14} into RNA in the present work is of the type discussed by Canellakis (133) and Heidelberger et al. (132) who used the same precursor.

Thus, even if association of c-RNA with the nuclear did not occur, the results of Figure X could not be taken as a proof that DNA has no effect on the synthesis of n-RNA since the experimental data give information only about the effect of DNA-ase on the incorporation of a radioactive precursor into RNA rather than RNA synthesis itself. Since incorporation in the present experiments is probably terminal and involves either an exchange reaction or direct attachment of a radioactives nucleotide to the end of the chain, such incorpor-

ation is not necessarily related to synthesis of RNA.

The present experiments shed some light on the effect of DNA-ase on the incorporation of radioactive precursors into RNA. However, the role of DNA in the synthesis of nuclear RNA is still far from settled and new experimental approaches are obviously required in order to make definite progress in the elucidation of this problem.

S U M M A R Y

1. Nuclei isolated from 20% rat liver homogenates were incubated with crystalline DNA-ase at 30° C for 20 minutes. Not more than 66-76% of total "apparent DNA" (diphenylamine reaction) could be removed by DNA-ase.

2. When the DNA-ase-treated nuclei were recombined with the cytoplasmic fractions and incubated with adenine-8-C^{14}, the specific activities of c-RNA and n-RNA in these reconstituted homogenates were decreased by 38-40% and 10-18% respectively by the removal of 66-76% of apparent DNA.

3. Failure to observe a large inhibition of the incorporation of radioactive adenine into n-RNA after DNA removal does not prove that DNA is not required for n-RNA synthesis.

4. Low concentrations of DNA-ase very slightly stimulate the incorporation of radioactive precursors into n-RNA.

5. The amount of n-RNA decreases and the amount of c-RNA increases during incubation of "reconstituted homogenates" when isolated nuclei are preincubated with crystalline DNA-ase.

PART III

THE NATURE OF THE DNA-ASE RESISTANT DIPHENYLAMINE CHROMOGEN

THE NATURE OF THE DNA-ASE RESISTANT DIPHENYLAMINE CHROMOGEN

It was shown in previous section that only 66-76% of "apparent DNA" (as determined by the diphenylamine (DPA) reaction) could be removed by DNA-ase, since the TCA extract of nuclei after DNA-ase treatment contained a chromogen which reacted with DPA.

Several other workers (120, 121, 118) have showed that a maximum of 60-80% of DNA in rabbit appendix and calf thymus nuclei can be removed by crystalline DNA-ase. Apparently they have assumed that the material remaining after DNA-ase treatment is DNA. For example, Sekiguchi and Sibatani (120) describe the remaining material as "the DNA remaining after DNA-ase treatment".

It is believed that this assumption is invalid and proof is presented in this section that there is no DNA remaining after treatment of the nuclei with DNA-ase. Instead, the data suggest that a new type of nucleic acid which produces a color with DPA is present.

EXPERIMENTAL

The procedure followed in the present experiments and described in this section was identical to the procedure used in the previous section. Nuclei isolated by centrifugation from 20% rat liver homogenates were incubated with crystalline DNA-ase in a solution containing 0.04 M $MgCl_2$ and 0.02 M phosphate buffer (PH 7) at 30° C for 20 minutes. The suspension mixture was then centrifuged at 0° C and the supernatant was discarded.

The resulting tissue was precipitated once with 5% TCA, washed three times with 5% TCA at 0° C, and then heated with 5% TCA at 90° C for 15 minutes. This TCA extract was used for all purposes of study in the present experiments, and it is referred to as the "TCA extract of the DPA chromogen, or simply as the "DPA chromogen" since it has only been studied in TCA extracts.

RESULTS AND DISCUSSION

Existence of the DPA Chromogen: After complete removal of DNA from nuclei with crystalline DNA-ase, a DPA chromogen still remained in the nuclei and could be extracted with hot 5% TCA from the tissues. The first evidence that is DPA chromogen was not DNA was the distinct difference in colors produced by pure DNA and the chromogen in the DPA reaction. The chromogen reacts with DPA to give a purple-violet color having a spectrum and absorption maximum different from the blue color obtained when pure DNA is heated with DPA (see Figure XII).

DNA has an absorption maximum at 600 millimicrons while the DPA chromogen has an absorption maximum at 560 millimicrons. This difference was enough to stimulate a detailed investigation of the nature of this fraction.

Mechanism of Action of DNA-ase: It was initially thought that in spite of the shift in the spectrum noted above, the DPA chromogen represented a type of DNA-ase-resistant DNA or a degradation product of DNA. Therefore, the literature on the mechanism of action of DNA-ase was reviewed and a brief summary of the information obtained is presented below.

Studies of the hydrolytic action of RNA-ase and DNA-ase on nucleic acids have led many workers to favour the existence of a "limit nucleotide" (137, 138) or core (139, 140). Kerr (140) definitely proved the existence of a core that could not be hydrolyzed by RNA-ase. The nature of this residue or "limit polynucleotide" was studied by many workers. Markham and Smith (141) and Volkin and Cohn (142) succeeded in separating the simpler components of this residue by ionophoresis and column chromatography respectively. They are unbranched di-, tri- and tetra nucleotides of different composition each having one pyrimidine nucleotide group per molecule and they are undiolyzable against water (143). But Markham and Smith (141) proved that the diffusibility of these oligonucleotides was increased when dyalized against solutions of high NaCl concentration.

The degradation of RNA by RNA-ase is very similar to the action of DNA-ase on DNA. Laskowski (144) and McCarty (145) succeeded in purifying DNA-ase from the pancreas and proving its specificity for DNA. It does not have any effect on RNA or any ribopolynucleotides. Tamm et al (146) have shown that the degree of DNA polymerization does not effect the rate and the extent of the activity of DNA-ase. However, it has been shown that the action of this enzyme is greatly deminished by the partial or total removal of purine group from DNA molecule (147, 146). The fact that apurinic acid (DNA treated with acid to remove all purines) (148, 149, 150) is completely resistant to the action of DNA-ase very clearly demonstrated the purine group requirement for the activity of DNA-ase.

Recently, Cohn (151) has shown that apurinic acid can easily be hydrolyzed by alkali and be proposed a mechanism for this hydrolysis. In each apurinated residue, the free 4'-hydroxyl resulting from the rupture of the furanose ring permits cyclization of the phosphate group with the new hydroxyl group. The degradation products of the above reaction would be cyclic 3'- and 4'- or 4'- and 5'- nucleotides as shown by Jones (152)

Overend et al (153) have demonstrated that DNA-ase degrades DNA into diffusible products of various molecular weights (153). Pyrimidine nucleotide groups were seemingly released preferentially, leaving a DNA-ase-resistant, purine rich residue. This result is very similar to the findings of Merrifield and Woolley (154) in relation to the specific cleaving action of RNA-ase on RNA.

Junger et al (155) have shown by the use of di-electric measurements that the breakdown of DNA passes through four stages. The first stage of this degradation is depolymerization or "disaggregation" of DNA, followed by splitting to smaller fragments in the second stage. In the third stage the enzyme is stimulated by the degradation products. In the fourth stage dialyzable groups appear. Varcauteren (156) has also studied the depolymerization of DNA and has suggested that it could not involve a rupture of internucleotide ester linkages

on the basis of questionable data on the precipitation of DNA and hydrolysis products with methylene blue.

It is obvious from the foregoing discussion that the study of DNA-ase action is rather difficult due to the innumerable fragments ranging from mononucleotides to polynucleotides (157) that are produced by enzymatic degradation.

Many workers have investigated the DNA-ase resistant polynucleotides obtained from the enzymatic degradation of DNA (139, 153, 158-162). Polynucleotides obtained from degradation of calf thymus nucleic acid had ratios of adenine to guanine, thymine to cytosine, and purine to pyrimidine that were higher than normal (143, 139). Little and Butler (163) have shown that the enzymatic degradation products of DNA are, on the average, tetranucleotides. On the other hand, Gordon and Reichard (164) from an electrophoretic study of the degradation products of DNA-ase, have demonstrated that the degradation fragments are dinucleotides such as adenine-cytosine and tri-nucleotides such as thymine and two cytosines.

Resistance of the DPA Chromogen to Hydrolysis by DNA-ase: In Figure X, it may be seen that the amount of apparent DNA (dephenylamine reaction) decreases to a constant level with increasing concentrations of DNA-ase. After this level is reached at 2 mg. DNA-ase/ml., increasing the DNA-ase concentration to 5 mg. of DNA-ase/ml. does not decrease the amount of apparent DNA.

Recently, Hurst (165) has shown that pancreatic DNA-ase has two separate actions. One is to degrade DNA molecules to oligonucleotides and the other is to hydrolyze these oligonucleotides to smaller fragments. He proved that Mn^{++} + EDTA at pH 8, rather than the usual conditions of Mg^{++} + phosphate buffer at pH 7 (146, 166) must be used in order to observe both actions of DNA-ase.

In the present experiment where only Mg^{++} was used, it was possible that DNA hydrolysis might be incomplete. Since, Hurst (165) showed that Mn^{++} is needed for full activity of DNA-ase, Mn^{++} was added to see if additional DPA

chromogen could be removed. The results in Table III show that the amount of DNA removed after one 20 minute incubation with DNA-ase in Mg^{++} + phosphate buffer at pH 7 (samples 5 and 6) was more than that removed by one incubation with DNA-ase in Mn^{++} + EDTA at pH 8 (samples 9 and 10).

When the same tissues were reincubated again under the same conditions for the same length of time, the quantity of DNA remaining after in the presence of Mg^{++} + PO_4 buffer at pH 7 (samples 7 and 8) remained unchanged. However, after the second incubation in the presence of Mn^{++} + EDTA at pH 8 (samples 11 and 12) the DNA decreased to the same minimum value obtained with Mg^{++} after the first incubation.

Cavalieri and Hatch (167) proved that DNA-ase activity is greatly inhibited by the degradation products of DNA. Failure of DNA-ase to hydrolyze all the DNA in the Mg^{++} system cannot be due to this inhibition in the present experiment since a second incubation after removing the first supernatant containing the degradation products did not decrease the amount of apparent DNA as discussed above.

Nature of DPA Chromogens: Many workers have investigated the colors given with diphenylamine by different sugars. Deriaz et al (168) proved that 2-deoxyribose groups of DNA are required for the color produced by DPA reagent. Overend et al (169) also studied the reaction and proved that 2-deoxyxylose gives a blue color having the same absorption maximum as DNA. 3-deoxyxylose and 2, 3-deoxyxylose produce a blue color with DPA which develops much slower than that produced with 2-deoxyribose (170). On the other hand, arabinal produced a blue color with DPA having an absorption maximum identical to 2-deoxyribose, but of different extinction coefficient (169).

It was shown by Mirsky (171) that when adenine and guanine deoxyribose nucleotides react with DPA, they give a color which is twice that of the equivalent amount of DNA. Dische (124) explained this by the fact that pyrimidine nucleotide groups do not show any significant color with the DPA

reagent. He also showed that thymidylic acid gives a faint blue color with an absorption maximum at 595 millimicrons and an extinction coefficient less than one-sixtieth of that of DNA. This indicates that only purine nucleotides react with DPA to give a color.

Substances that interfere with the DPA reaction: In the present experiments the TCA extraction method of Schneider (172) has been used for the isolation of DNA from tissues. It was thought the DPA chromogen obtained in the present experiments after removal of the DNA with DNA-ase could be a contaminant since DPA gives color with many different substances other than DNA such as, unknown protein substances (173) and lipid-soluble substances in plants (174).

Barnum and Huseby (175) found that TCA extracts contain phospho-proteins which react with DPA. Dische (124) relates that Pirie (173) found unknown protein-bound substances that gave a purple color with DPA which has an absorption maximum at 530 millimicrons-Lipid-soluble substances found in plants gave a purple color having an absorption maximum at 560 millimicrons (174). However, a literature search provided no evidence for the existence of these latter lipid-soluble substances in animal tissues.

Finamore (176) proved the existence of a protein in the amphibian egg that could be extracted by hot 5% TCA and that gave a color with DPA. This protein could be precipitated out of the TCA extract by chilling at zero degrees for several hours. Attempts to remove the DPA chromogen from TCA extracts by this method in our laboratory were unsuccessful. It may be seen from Table IV that the amounts of DNA determined by the DPA reactions on identical samples, with and without chilling for 20 hours at 0° C, were nearly the same. In addition, the absorption maxima of all samples with and without chilling were at 600 millimicrons.

Glycogen and RNA are not responsible for the color of the DPA chromogen, since when they were hydrolyzed with 5% TCA at 90° C for 15 minutes and when the resulting extract was tested with the DPA reagent, neither substances produced any color.

The nature of the chromogen could be sugar other than 2-deoxyribose. Therefore, a series of 18 locally available sugars (listed in Table V) was reacted with DPA under the usual conditions by heating 1 ml. of the sample (2 mg. sugar/ml.) and 2.5 ml. of the diphenylamine solution for 20 minutes at 100° C in boiling water. Unfortunately, deoxysugars (other than rhamnose and fucose) were not available.

Most of the sugars gave no color with diphenylamine. However, xylose, arabinose and lyxose gave an extremely faint green color while ribose gave a faint blue grey color. Levulose, sorbose and raffinose gave very deep blue grey, clear blue and blue-green colors respectively.

None of these matched the color given by the DPA chromogen. It may be seen from Figure XII, that pure DNA has a sharp absorption maximum at 600 millimicrons. The DPA chromogen, on the other hand, has a broad absorption maximum extending from 550 to 570 millimicrons with the maximum at 560 millimicrons the spectra of levulose, sorbose and raffinose after reaction with DPA each had three almost identical maxima with the extinction for each peak decreasing from levulose to sorbose to raffinose. These maxima were at about: 370, 520 and 645 millimicrons for levulose; 360, 520 and 655 millimicrons for raffinose. Thus, the DPA chromogen with a single maximum at 560 millimicrons is clearly none of these three sugars nor is it similar to the curve of pure DNA.

Proof of the Absence of Deoxyribose in the DPA Chromogen: It was possible that the color given by the DPA chromogen was due to deoxyribose with some impurities that changed the characteristic color of the sugar. The DPA chromogen and pure DNA were heated with the Stumpf cysteine-H_2SO_4 reagent

(177) and the absorption spectra were determined as shown in Figure XIII. Pure DNA gave a pink color having an absorption maximum at 490 millimicrons, while the DPA chromogen failed to produce any color with cysteine-H_2SO_4 or to show any detectable shoulder at 490 millimicrons. This clearly proves that the DPA chromogen does not have any detectable deoxyribose.

In addition, the DPA chromogen and pure DNA were tested with the tryptophan-perchloric acid reagent (178) and the absorption spectra of these samples were determined as shown in Figure XIV. Again, no rise could be detected in the spectrum of the DPA chromogen at the DNA absorption maximum of 500 millimicrons. This confirms the fact that the DPA chromogen does not contain deoxyribose.

The Non-Extractability of the DPA Chromogen with NaCl: It was felt that the DPA chromogen might possibly be a nucleic acid*, and an attempt was made to purify the DPA chromogen on the basis of this assumption. Since nucleic acids are extracted from tissue by heating with 10% NaCl (179-183), DNA was removed from the tissue by DNA-ase, the tissue was precipitated with cold 5% TCA, washed 3 times with cold 5% TCA, and then extracted with 10% NaCl at 100° C for 20 minutes. It was expected that if the chromogen were a nucleic acid, it would be extracted into the NaCl and could be precipitated by ethanol. However, the nucleic acid precipitate obtained in this way gave no color with DPA.

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* This assumption was originally based on the nucleic acid-like ultraviolet spectra of TCA extracts of tissue from which DNA had been removed by DNA-ase. (It was not realized at the time that RNA was still present).

Since the chromogen was not present in the nucleic acid fraction, it was either present in the supernatant after alcoholic precipitation of the nucleic acids or was still bound to the tissue. The latter possibility was tested, still assuming that the material might be a nucleic acid, by using the standard nucleic acid extraction technique of Schneider (172) (heating the NaCl-extracted residue with 5% TCA at 90° for 15 minutes). When this hot TCA extract was tested in the DPA reaction, it gave a color typical of the DPA chromogen.

This finding was of considerable importance because it simplified the preparation of the DPA chromogen and eliminated the need for DNA-ase. It is now possible to obtain the chromogen simply by extracting RNA and DNA with 10% NaCl, leaving a residual tissue still containing the tightly bound chromogen.

The procedure now used for obtaining extracts of the chromogen is as follows: After isolating nuclei from 20% rat liver homogenates, the acid-soluble fraction is removed by precipitating the tissue with cold 5% TCA and washing once with TCA at 0° C. The tissue is heated twice with 10% NaCl at 100° C for 20 minutes to remove RNA and DNA. The resulting tissue is then heated with 5% TCA at 90° C for 15 minutes to extract the DPA chromogen. From the data given in Figure XV it may be seen that the spectrum of the chromogen after reaction with DPA is almost identical regardless of whether the DNA has been removed by incubation with DNA-ase or by two extractions with 10% NaCl at 100° C.

In addition, the amount of DPA chromogen obtained after removal of nucleic acid with 10% NaCl at 100° C. (0.98 and 0.97 mg. equivalent DNA/gm. tissue in one experiment) is identical with the amount of DPA chromogen obtained after removal of DNA with DNA-ase (0.97 and 0.95 mg/gm. tissue in the same experiment). These values are expressed in terms of mg. of DNA that would

give the same optical density in the DPA reaction.

It has also been shown that the intensity of the DPA chromogen color remains constant regardless of the number of NaCl extractions of the tissues (up to 4 extractions).

The inability to extract the DPA chromogen from tissue by 3-4 extractions with cold 5% TCA or by 4 extractions with 10% NaCl at 100° C indicates that the DPA chromogen is very tightly bound to protein. However, it is not possible to conclude that it is a polymeric material, since small molecules can be bound as tightly as large ones.

Ultraviolet Absorption Spectra of the DPA Chromogen: It may be seen in Figure XVI that the two ultraviolet absorption spectra of the TCA extract of the DPA chromogen and highly polymerized DNA at acidic pH are identical and that they have the same absorption maxima at 260 millimicrons. This indicates that the DPA chromogen is composed of purine and pyrimidine bases that absorb at 260 millimicrons.

Presence of Purine and Pyrimidine Bases in the DPA Chromogen: Identification of the different components present in the DPA chromogen was then undertaken. In a preliminary experiment the TCA extract of the DPA chromogen was extracted with ether to remove TCA. The resulting solution was put on a Dowex-1-formate column and the hydrolysis products were separated by gradient elution as described by Hulbert et al. (185).

The results of this elution showed that the DPA chromogen contains multiple components. One fraction was not absorbed by the Dowex-1-formate and it was eluted with water. The other components remaining on the column were eluted gradiently as three major peaks. Using a 200 ml. of H_2O in the mixer, these peaks were eluted by the sequential addition of 125 ml. of 0.8 N formic acid, 285 ml. of 4 N formic acid and 100 ml. of 8 N formic respectively to the resevoir. The first two peaks appeared to be composed of a series of multiple sub-peaks. Unfortunately, it is not certain whether these variations in optical

density from tube to tube were due to technical difficulties in reading the samples or to the actual occurrence of many small overlapping peaks, each representing a different degradation product. However, it was evident that the behavior of the different components was identical with what would be expected if a nucleic acid had been hydrolyzed with TCA.

It is well established that the acid hydrolysis products of nucleic acid are free purines and pyrimidine mononucleotides or polynucleotides depending on the strength of acid used, the length of hydrolysis, and the temperature at which the hydrolysis is performed (186, 187, 188).

When the water eluate was chromatographed on Whatman No. I filter paper in a solvent of n-butanol (84%) and water (16%) at 30° C for 24 hours as described by Markham and Smith (189), adenine was definitely identified as one of the components. Guanine could not be identified due to impurities present in the water eluate. In addition, pure guanine chromatographed simultaneously with a sample of the water eluate had an R_f of zero. However, guanine was later definitely proved to be present in the DPA chromogen (discussed later).

The first two peaks eluted with acid were thought to be pyrimidine-containing oliganucleotides. These fractions were combined and evaporated to dryness under vacuum. The resulting residue was dissolved in 12 N PCA and heated in a water bath at 100° for 90 minutes to obtain a quantitative yield of all the bases as described by Marshak and Vogel (190, 191).

The resulting solution was evaporated to dryness, spotted on paper and chromatographed using a mixture of n-butanol (84%) and water (16%) as a solvent (189). Although impurities present prevented a positive proof that pyrimidine bases were present on the chromatogram, it was clear that spots of known cytosine and uracil coincided with a part of the ultraviolet absorbing material present in the unknown sample.

Since it was shown by this preliminary experiment that purines and pyrimidines might be present in the DPA chromogen direct identification of the bases present in the DPA chromogen was undertaken.

A TCA extract of the DPA chromogen from 22.5 grams of liver was extracted four times with ether to remove the TCA and the aqueous phase was evaporated to dryness under vacuum. The residue was heated in 12 N PCA (190, 191) for 90 minutes at 100° C in boiling water to liberate all the purine and pyrimidine bases present in the DPA chromogen. The PCA was removed by precipitation with KOH as KCl O_4 (potassium perchlorate) at 0° C and the bases were obsorbed on a Dowex-50-H^+ column (6 cm. high, 1 cm. diam.). Four distinct peaks were separated by gradient elution using 200 ml. of water in mixer and 400 ml. of 6 N HCl in the reservoir. The first peak was eluted with water. The optical density of the fraction was read on a Zeiss spectrophotometer and E_{275}/E_{260} were found to be 0.61, 1.14, 0.75 and 0142 respectively. The four fractions were then spotted on paper as described before and chromatographed with a solvent containing n-butanol (84%) and water (16%) at 30° for 20 hours. The resulting spots were eluted from the paper by water and rechromatographed using the same procedure. The comparative R_f (comparative R_f = $\frac{\text{distance moved by unknown spot}}{\text{distance moved by known base}}$) of the samples were determined and found to be 0.98, 1.03, 0.98, 0.99 and 0.99 for uracil, thymine, cytosine, guanine and adenine respectively. It is clear that all five major bases usually present in nucleic acids are found in the DPA chromogen. However, thymine and uracil gave lighter spots than the other bases, indicating that they are found in smaller amounts in the DPA chromogen. The thymine spot was barely, but definitely detectable.

Stability of the DPA Chromogen to Alkaline Hydrolysis and PO_4 Content: After NaCl extraction of nuclei, three separate samples of the NaCl-extracted nuclei were heated with 0.1 N KOH, 0.2 N KOH and distilled water (PH 7) at 80° C for 10 minutes. KOH was then nutralized with TCA at 0° C and the

resulting residues treated as described under legend for Table VI and the DPA chromogen contents of the three samples were determined by the DPA reagent. It may be seen from Table VI that the DPA chromogen contents of the three differently treated samples were almost the same. This indicates that the DPA chromogen is neither RNA nor an apurinated residue of DNA since apurinic acid is hydrolyzed when heated with alkali (151).

In addition, the phosphorus content of the DPA chromogen was determined and found to be 9.5% and 10.25% in two independent experiments. The phosphorus content of pure DNA is known to be approximately 9% (192). It will be desirable to determine nitrogen content of the DPA chromogen and calculate accurate $N/_P$ ratios in the course of future works.

Incorporation of Thymine into the DPA Chromogen: After demonstrating that thymine is present in the DPA chromogen it was desirable to investigate the incorporation of tritiated thymidine into the DPA chromogen in vivo. Hecht and Potter (193, 194) demonstrated that partially hepatectomized rats incorporate tritiated thymidine into DNA of the regenerating liver, beginning 18 hours after partial hepatectomy. In the present experiment, a partially hepatectomized rat was injected with triatiated thymidine using procedure described under Figure XVII. After sacrificing the rat, the isolated nuclei were incubated with DNA-ase and the specific activities of DNA and the DPA chromogen were determined as shown in Figure XVII.

The specific activity of DNA after correction for presence of DPA chromogen is 710 c.p.m./mg. DNA while the specific activity of DPA chromogen is 82 expresse in terms of c.p.m./mg. of DNA that would give the same optical density in the DPA reaction. Triatiated thymine was incorporated 8.5 times higher into DNA than into DPA chromogen. This difference in thymine-incorporation might reflect either low thymine content of DPA chromogen or higher degree of biochemical stability than DNA itself.

It may be seen that the specific activity of DNA decreases as DNA is removed by DNA-ase until the specific activity becomes constant no matter how much the DNA-ase concentration is increased. The data show that when DNA is completely removed, the specific activity of the remaining DPA chromogen reaches a small but significant constant value. It is obvious then, that the DPA chromogen is quite different from the DNA-ase-labile fraction.

Presence of the DPA chromogen in the Cytoplasm: In preliminary experiments the cytoplasmic fraction of 20% rat liver homogenates were washed twice with TCA at 0° and heated twice with 10% NaCl at 100° for 20 minutes. The NaCl-extracted tissues were then heated with 5% TCA at 90° for 15 minutes. When the resulting TCA extracts were tested with diphenylamine reaction, the color characteristic of the "DPA chromogen" in the nucleus was observed. In one experiment the DPA chromogen content of the cytoplasm was determined on Klett spectrophotometer and the results were 0.19 and 0.21 mg./gm. tissue compared to 1.06 mg. and 1.1 mg./gm. tissue in the nucleus in the same experiment. (Values are expressed in terms of the mg. of pure DNA which give an equivalent color). The absorption spectrum of the cytoplasmic DPA chromogen was determined and the absorption maximum was at 560 mu (identical with that of the nuclear fraction).

Interpretation of the Data: It is evident from the data that the DPA chromoge is composed of adenine, guanine, cytosine, uracil and thymine, phosphate and probably a sugar of unknown nature different from both ribose and deoxyribose. The DPA chromogen gives no color with cysteine-H_2SO_4 or tryptophan-perchloric acid reagents while DNA gives definite color. Reaction of the DPA chromogen with orcinol gave only a dirty brown discoloration and not the green obtained with RNA. The DPA chromogen has almost an identical ultraviolet spectrum with that of DNA with the same absorption maximum at 260 millimicrons.

The DPA chromogen is not hydrolyzed by pancreatic DNA-ase or alkali nor is it extracted from tissue by heating with 10% NaCl or alkali. In one gram of liver there is 0.95-1.58 mg. in nuclei and 0.19 to 0.21 mg. DPA chromogen in the cytoplasm in terms of mg. of DNA that give an equivalent color. Tritiated

thymine is incorporated into the DPA chromogen to small but significant extent. Its specific activity is one-ninth of the specific activity of DNA (corrected for DPA chromogen content) in the same experiment.

The foregoing properties of the DPA chromogen can best be explained if it is assumed that this chromogen is a nucleic acid-like material tightly bound to protein. Proof of this assumption will require the demonstration of nucleotide-like monomers containing a purine or pyrimidine base, the chromogenic moiety and phosphate all in one intact unit. Hydrolysis with mild acid or with enzymes should provide such fragments. The chromogenic moiety should then be identified. Since both chromogen and deoxyribose give a color with diphenylamine, the DPA chromogen may be deoxysugar. However, the possibility that it has an entirely different type of structure cannot be excluded, since the chromogenic unit gives no color with cystein-sulfuric acid or tryptophane-perchloric acid reagents.

No direct evidence has yet been reported to prove that DNA is the exclusive carrier of genetic information or to establish the means by which this genetic information is transferred to the cytoplasm. In addition, modern concepts of the genes consider the biochemical stability of DNA as one of the main proofs that DNA is the genetic material of the body. However, it has been shown in in this paper that the DPA chromogen is very tightly bound to protein and is resistant to DNA-ase and alkaline hydrolysis. In addition it does incorporate thymidine to a very smaller extent than DNA (although this may be due to low thymine content since other precursors have not yet been tested).

It is therefore possible that the DPA chromogen is genetic material which may plays as important a role as DNA. Allfrey and Mirsky (121) and Sekiguchi and Sibatani (120) suggest that "the DNA-ase-resistant fraction of DNA" (which is almost certainly identical to the DPA chromogen) might possibly control protein and RNA synthesis. The DPA chromogen might also be active in transferring genetic information to the cytoplasm since it has been shown in this paper that the DPA chromogen is present in the cytoplasm. Such a possibility would explain the

the failure of the complete DNA removal to significantly inhibit the incorporation of radioactive precursors into n-RNA.

SUMMARY

1. When nuclei isolated from 20% rat liver homogenates are incubated with crystalline DNA-ase, a diphenylamine chromogen (DPA chromogen) remains in the nuclei which can be extracted by heating with 5% TCA for 15 minutes at 90° C. The chromogen has an absorption maximum at 560 millimicrons rather than the 600 millimicrons maximum given by pure DNA.

2. The DPA chromogen could not be extracted by 10% NaCl (100° C for 20 minutes) nor with 0.2 N KOH (80° C for 10 minutes). A very similar absorption spectra was obtained with the TCA extract of the DPA chromogen, whether DNA has been removed from the nuclei by heating with 10% NaCl or by treatment with DNA-ase.

3. No detectable deoxyribose is present in the DPA chromogen since it failed to give any color with cysteine-sulfuric and or with tryptophan-perchloric acid reagents. In addition, ribose is not present, since the DPA chromogen did not give a green color with the orcinol reagent. A series of 18 common sugars was tested with DPA, and only levulose, raffinose and sorbose gave blue colors. However, all these were visibly different from that of the DPA chromogen.

4. The ultraviolet spectra of the DPA chromogen and pure DNA were found to be almost superimposable between 240 and 300 millimicrons with absorption maxima at 260 millimicrons in both cases. All pyrimidine and purine bases are present in the DPA chromogen. Behavior of the acid hydrolysis products of the DPA chromogen on Dowex-1-formate column in a preliminary experiment was similar to that expected of a nucleic acid in that adenine was eluted in the water wash and three major fractions remained on the column and were eluted with acid.

5. Expressing values as mg. of DNA that give an equivalent color with DPA there is 0.95-1.58 mg. of the DPA chromogen in the nucleus and 0.19-0.21 mg. in the cytoplasm per gram of rat liver tissue. Phosphate content was found to be 9.5-10.2% in two separate experiments.

6. A small but significant incorporation of radioactive thymidine (82 c.p.m./mg. DNA) into the DPA chromogen was obtained when tritiated thymidine was injected 18 hours after partial hepatectomy and the rat was sacrified 5 hours later. DNA had a specific activity 8.5 times higher in the same experiment.
7. These findings prove that the DPA chromogen is a nucleic acid-like material, tightly bound to proteins through unknown linkages which can not be broken by boiling with 10% NaCl or heating with 0.2 N alkali. However, the DPA chromogen can be hydrolyz and extracted with hot 5% TCA. The nature of the chromogenic moiety and its linka to the phosphate, purine and pyrimidine moieties require further investigation.

Figure 1. Effect of Non-Crystalline DNA-ase on the Incorporation of Orotic Acid-6-C^{14} into RNA of Whole Rat Liver Homogenate.

2.5 ml. aliquots of 2% rat liver homogenates in 0.25 M sucrose were incubated with different concentration of non-crystalline DNA-ase (indicated on the abscissa) using standard incubation procedure (see text). After incubation, the nuclear and cytoplasmic fractions were separated by differential centrifugation and RNA was extracted from the two fractions. The specific activities of RNA were determined and are given in terms of c.p.m./mg. RNA on the ordinate. Every point at zero concentration of DNA-ase represents the average of triplicate samples, which were in good agreement.

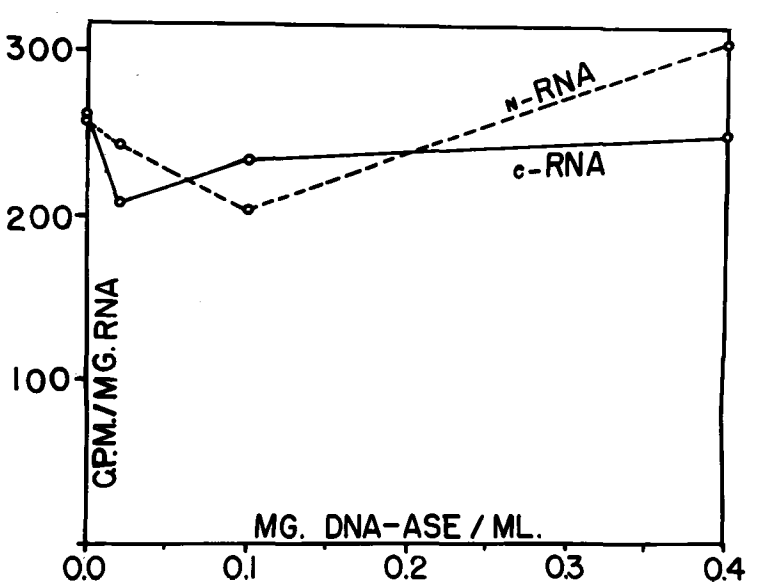

Figure II. Effect of Non-Crystalline DNA-ase on the Incorporation of Orotic Acid-6-C^{14} into RNA of a Rat Liver Homogenate after Preincubation with DNA-ase at 0° C.

2.5 ml. aliquots of 20% rat liver homogenates were preincubated with non-crystalline DNA-ase at 30° C for 3 hours and then incubated as described under "experimental" (see text). The specific activities of RNA were determined and are given in terms of c.p.m./mg. RNA on the ordinate. Every point on this graph represents the average of duplicate determination of specific activity of RNA.

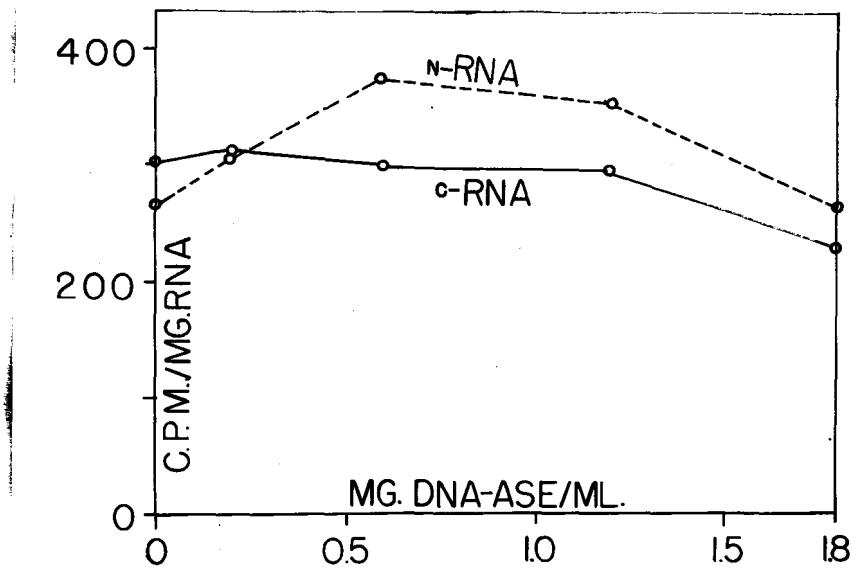

Figure III. Effect of Preincubation at 30° C on the Incorporation of Orotic Acid-6-C^{14} into RNA of Rat Liver Homogenate.

2.5 ml. aliquots of 2% rat liver homogenate were preincubated at 30° C for different intervals of time (indicated on the abscissa). After preincubation, the homogenate was incubated with orotic acid-6-C^{14} under standard incubation conditions (see text). The c-RNA and n-RNA were isolated and the specific activity was determined as c.p.m./mg. RNA (indicated on the ordinate) in two different experiments (solid and dotted lines). Points at zero time represent the average of duplicate samples.

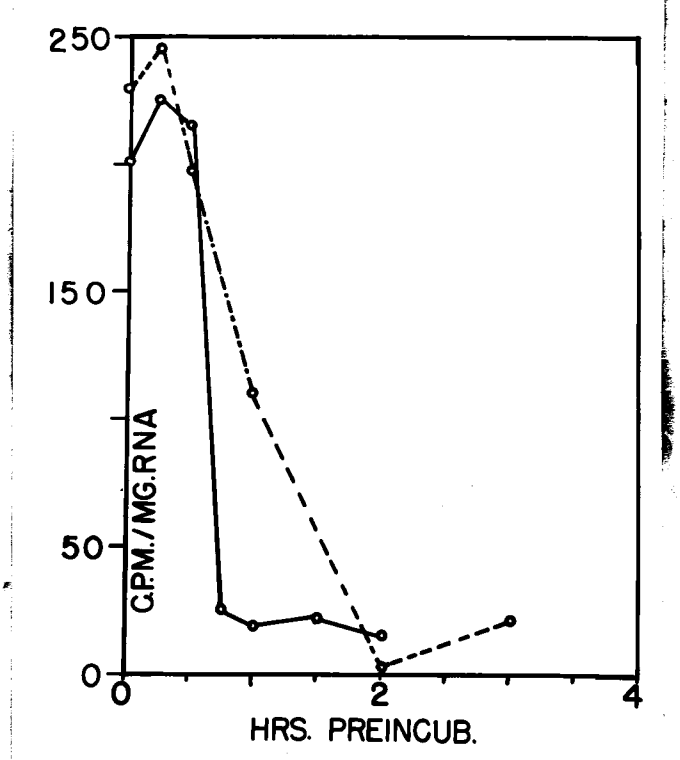

Figure IV. Effect of Preincubation with Non-Crystalline DNA-ase on the Incorporation of Orotic Acid-6-C^{14} into RNA in Whole Rat Liver Homogenates.

2.5 ml. aliquots of 20% homogenates of rat liver were preincubated with non-crystalline DNA-ase at 30° C for 30 minutes and then were incubated using the standard incubation procedure (see text). The specific activities as c.p.m./mg. RNA of c-RNA and n-RNA were determined and are given on the ordinate. The different concentrations of DNA-ase as mg./ml. are indicated on the abscissa. Every point at zero concentration of DNA-ase represents the average of duplicate samples.

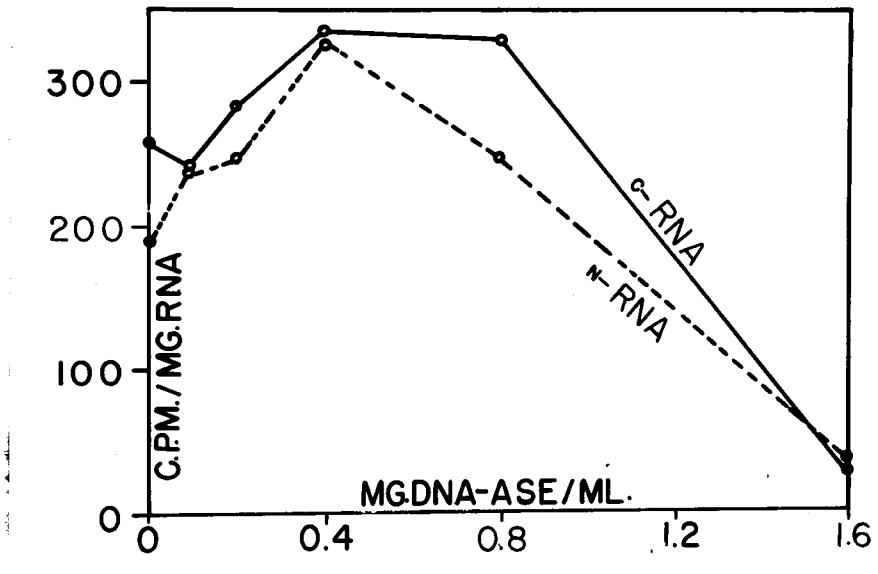

Figure V. Effect of Preincubation with $MgCl_2$ for Different Intervals of Time on the Incorporation of Orotic Acid-6-C^{14} into RNA of a Rat Liver Homogenate.

2.5 ml. aliquots of a 20% rat liver homogenate were preincubated with 0.006 M $MgCl_2$ (dotted line) or 0.003 M $MgCl_2$ (solid line) at 30° C for different intervals of time (indicated on the abscissa) in 50 ml. flasks. After preincubation, the homogenates were incubated with orotic acid-6-C^{14} under standard incubation conditions (see text), the c-RNA and n-RNA were isolated, and the specific activity was determined as c.p.m./mg. RNA (indicated on the ordinate). Every point at zero time represents the average of duplicate samples.

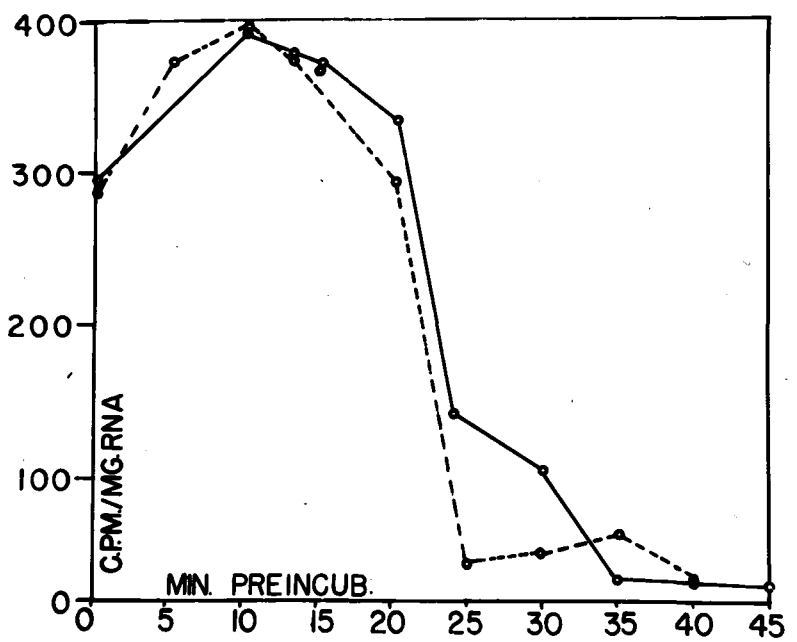

Figure VI. Effect of Crystalline DNA-ase on the Incorporation of Orotic Acid-6-C^{14} into RNA after Preincubating Rat Liver Homogenates with 0.006 M $MgCl_2$.

2.5 ml. aliquots of 20% homogenates of rat liver were preincubated with 0.006 M $MgCl_2$ and crystalline DNA-ase at 30° C for 15 minutes and then incubated under the standard conditions (see text). The specific activities as c.p.m./mg. of n-RNA and c-RNA are given on the ordinate and the different concentrations of DNA-ase are indicated on the abscissa.

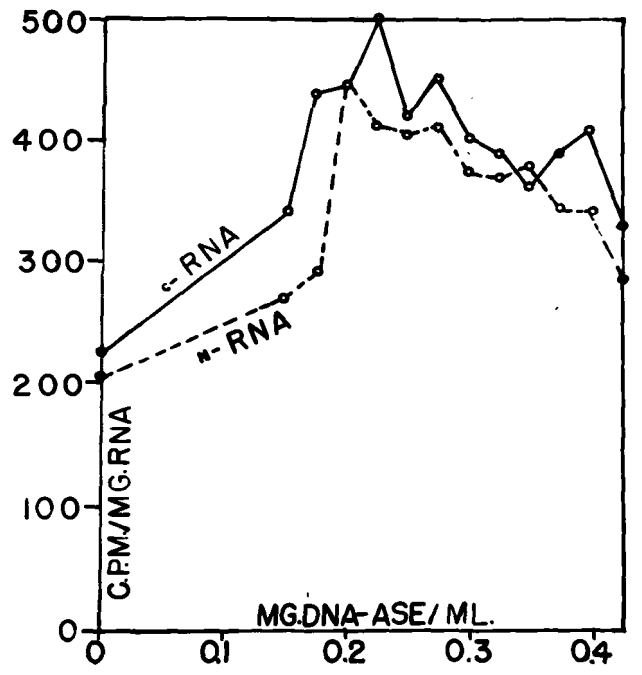

Figure VII. Effect of Crystalline DNA-ase on the Incorporation of Orotic Acid-6-C^{14} into RNA of Non-Preincubated Rat Liver Homogenate.

2.5 ml. aliquots of 20% rat liver homogenate in 0.25 M sucrose were incubated with different concentrations of DNA-ase as indicated on the abscissa using the standard incubation procedure (see text). The nuclear and cytoplasmic fractions were isolated by differential centrifugation and the specific activities of c-RNA and n-RNA were determined and are given in terms of c.p.m. /mg. RNA on the left ordinate. Every point at zero concentration of DNA-ase represents the average of triplicate values for specific activity. The DNA content of nuclei from 2.5 ml. aliquots of homogenates treated identically in separate flasks was determined in the same experiment as mg. DNA/gm. tissue (indicated on the right ordinate). The point at zero concentration of DNA-ase represents the average of duplicate determinations of DNA content.

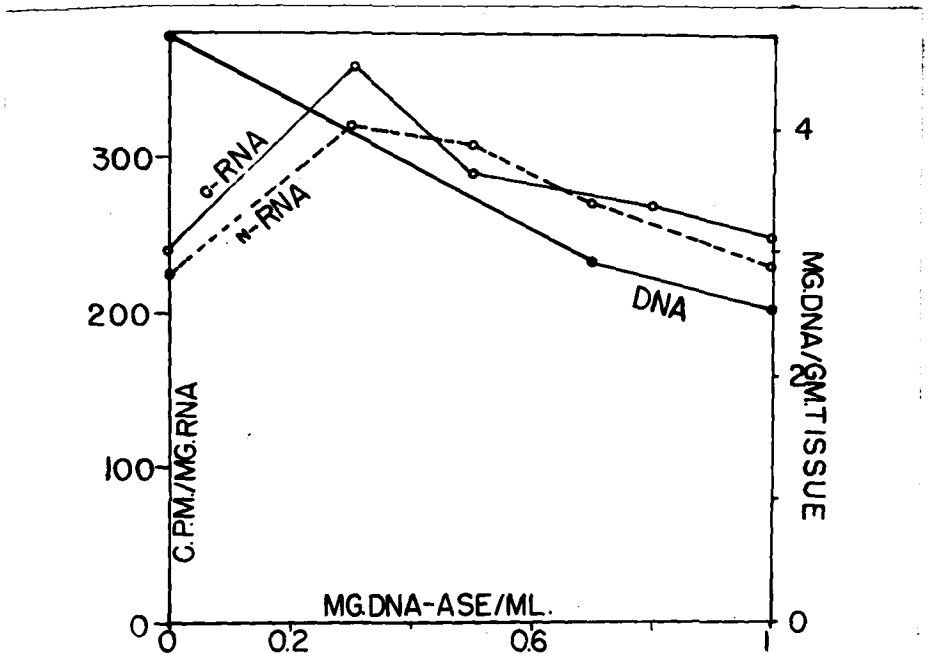

Figure VIII. Effect of Preincubation of Isolated Nuclei on the Incorporation of Adenine-8-C^{14} into RNA of Reconstituted Homogenates of Rat Liver.

Nuclei were isolated from 20% rat liver homogenates by the standard procedure (see text) and preincubated with $MgCl_2$ (0.02 M) and pH 7.0 phosphate buffer (0.02 M) at 30° C for different periods of time (indicated on the abscissa). The isolated nuclei were then recombined with the corresponding cytoplasmic fractions and incubated under standard incubation conditions (see text). The specific activities of c-RNA and n-RNA were determined and the results are given on the ordinate in terms of c.p.m./mg. RNA for two separate experiments (open and close circles). Points at zero time represent the average of duplicate samples.

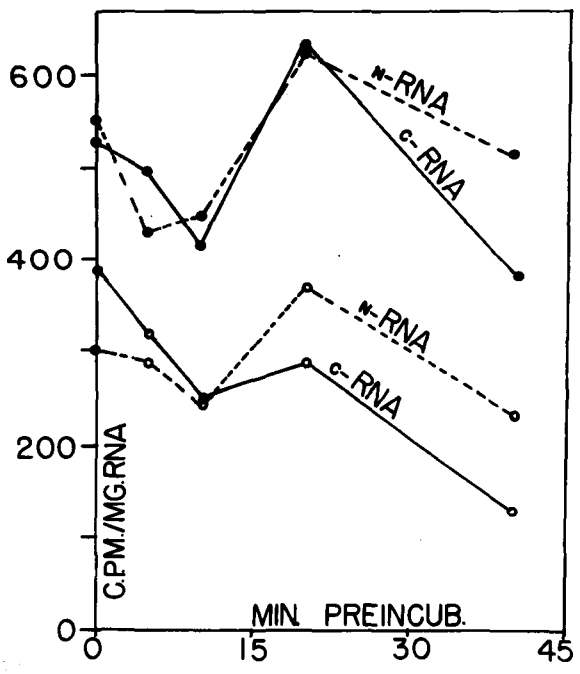

Figure IX. Effect of Preincubating Isolated Nuclei with Magnesium prior the Incorporation of Adenine-8-C^{14} into RNA of a Reconstituted Rat Liver Homogenate.

Nuclei were isolated from 20% rat liver homogenate by the standard procedure (see text) and incubated with pH 7.0 phosphate buffer (0.02 M) and different concentrations of $MgCl_2$ (indicated on the abscissa) at 30° C for 20 minutes. After incubation, nuclei were recombined with the cytoplasmic fractions and incubated under standard incubation conditions (see text). c-RNA and n-RNA were isolated and their specific activities were determined in terms of c.p.m./mg. RNA (given on the ordinate.

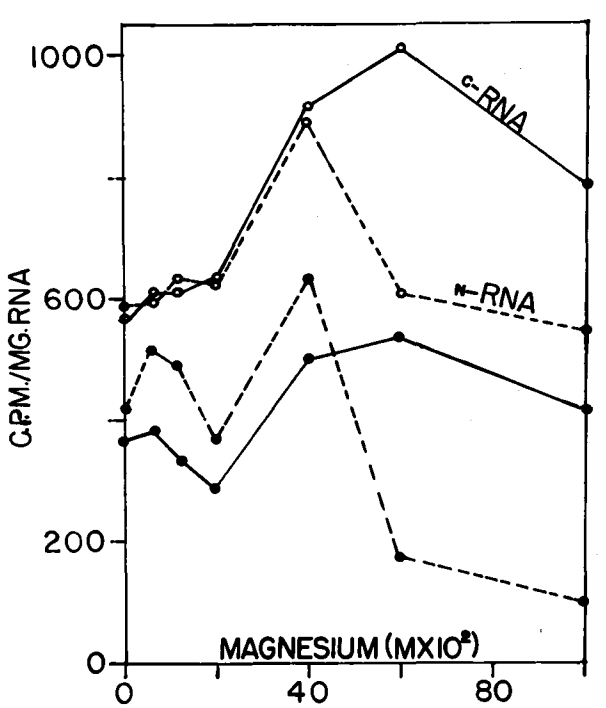

Figure X. Effect of Preincubation of Isolated Nuclei with DNA-ase on the Subsequent Incorporation of Adenine-8-C^{14} into RNA of a Reconstituted Homogenate.

Isolated nuclei from 6 ml. aliquots of 20% rat liver homogenates in 0.25 M sucrose solution were preincubated for 20 minutes in 12 ml. tubes with solutions containing DNA-ase (concentrations indicated on the abscissa), $MgCl_2$ (0.04 M) and pH 7.0 phosphate buffer (0.02 M). After incubation the DNA-ase-treated nuclei were recombined with the cytoplasmic fractions from which they were separated initially. 2.5 ml. aliquots of the reconstituted homogenates were incubated under standard conditions (see text) in 50 ml. erlenmyer flasks at 30° C. for 45 minutes. c-RNA and n-RNA were isolated and their specific activities were determined three separate experiments (open and closed circles and a cross). Results are given in the bottom graph with specific activity of RNA in terms of c.p.m./mg. RNA on the ordinate. Every point at zero concentration of DNA-ase represents the average of duplicate determinations of specific activity.

Nuclei were reisolated from 2.5 ml. aliquots of the same reconstituted homogenates by centrifugation at 600 x g. DNA was extracted from the nuclei and determined by the diphenylamine reaction. The DNA concentration (mg. DNA/gm. tissue) is given in the top graph. Every point at zero concentration of DNA-ase represents the average of duplicate determinations of DNA content.

Figure X

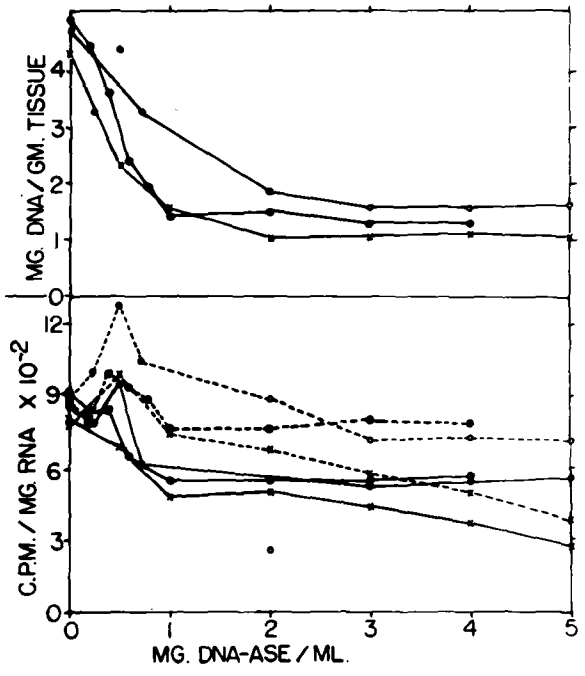

Figure XI. Effect of Preincubation of Isolated Nuclei with DNA-ase on the Relative Amounts of Nuclear and Cytoplasmic RNA.

Values for amount of n-RNA and c-RNA and total RNA (as Mg. RNA/gm. tissue) were calculated from the experiments presented under Figure X.

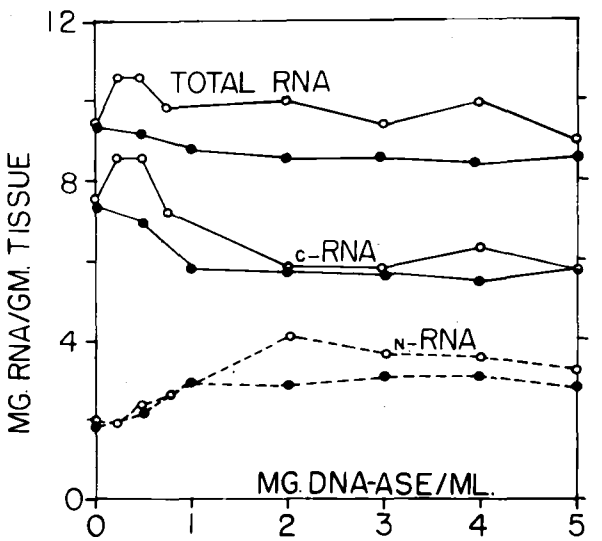

Figure XII. Absorption Spectra of the DPA Chromogen and Highly Purified DNA after Reaction with Diphenylamine.

Nuclei isolated from 20% rat liver homogenates were incubated with DNA-ase as described under "experimental". The nuclear pellets were washed three times with 5% TCA at 0° C. and the DPA chromogen was extracted from the nuclei with hot 5% TCA at 90° C for 15 minutes. One ml. aliquots of the TCA extract (0.5 gm. tissue/ml.) were heated with diphenylamine (124) and the absorption spectrum was determined on a Zeiss spectrophotometer. One ml. aliquots of a solution of highly polymerized DNA (0.25 mg./ml.) were reacted with diphenylamine and used for determining the spectrum of DNA.

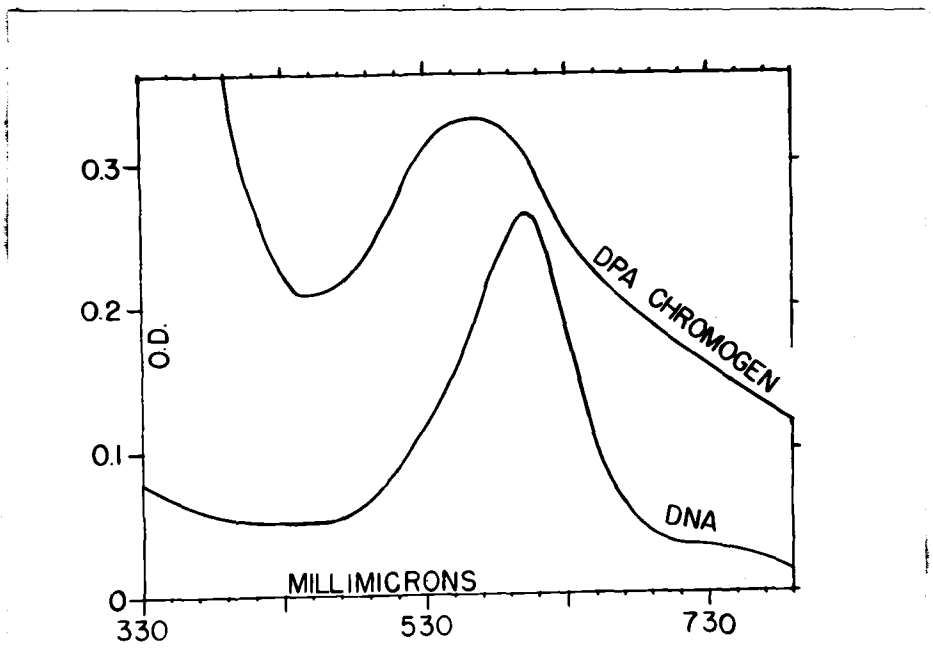

Figure XIII. Absorption Spectra of the DPA Chromogen and Highly Polymerized DNA after Reaction with the Cysteine-Sulfuric Acid Reagent.

TCA extracts of DPA chromogen were prepared as described in legend for Figure XVI. 0.5 ml. of the TCA extract (0.5 gm. tissue/ml.) and 0.25mg. of DNA were heated with a cysteine-sulfuric acid reagent (177) and the absorption spectra were determined on a Zeiss spectrophotometer.

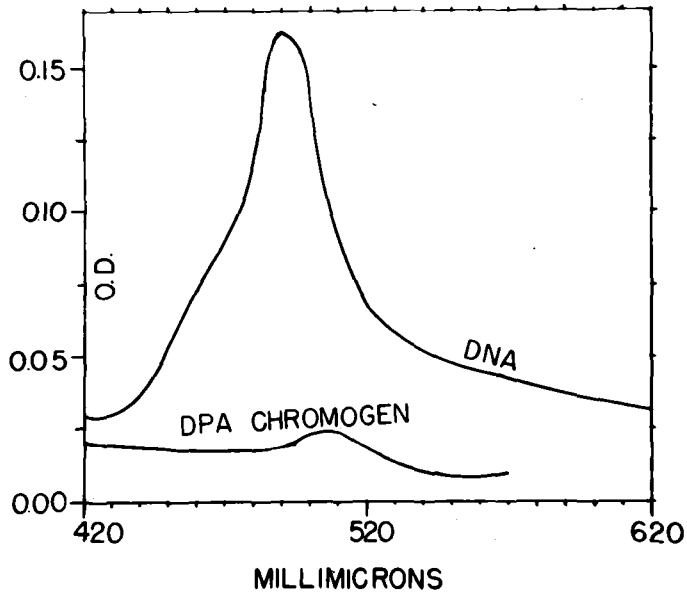

Figure XIV. The Absorption Spectra of the DPA Chromogen and Highly Polymerized DNA after Reaction with the Tryptophan-Perchloric Acid Reagent.

TCA extracts of the DPA chromogen were prepared as described under legend for Figure XVI.

One ml. aliquots of this extract (0.5 gm. tissue/1 ml.) were tested with the tryptophan-perchloric acid reagent (178) and the absorption spectrum was determined on a Zeiss spectrophotometer. 0.5 mg. of highly polymerized DNA was used for determining the spectrum of DNA.

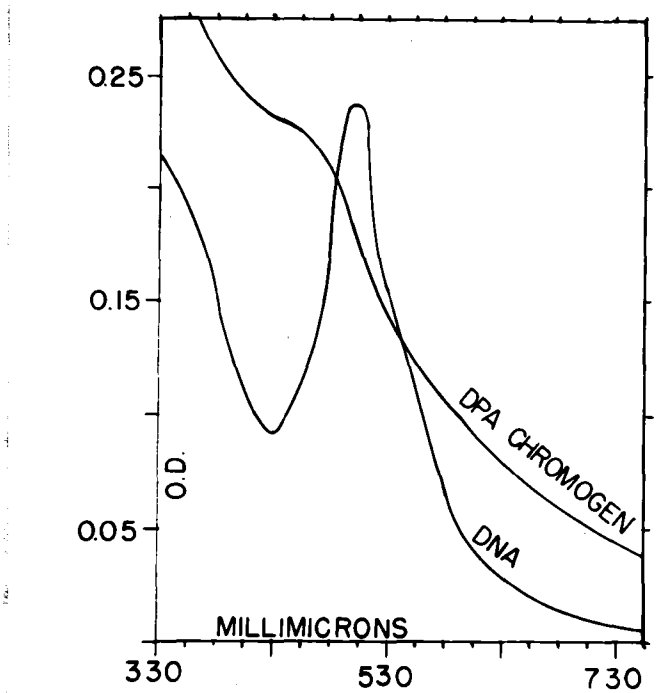

Figure XV. Comparison of the Absorption Spectra of the DPA Chromogen after DNA-ase Treatment and NaCl Extraction.

Nuclei were isolated from 2.5 ml. aliquots of 20% rat liver homogenates. One nuclear pellet was incubated with DNA-ase (curve 1) using the standard incubation procedure (see text). A second nuclear pellet was extracted twice with 10% NaCl at 100° C for 20 minutes (curve 3). A third nuclear pellet was first incubated with DNA-ase and then extracted twice with 10% NaCl (curve 2). These three pellets were then heated with 5% TCA at 90° for 15 minutes to extract the DPA chromogen. The TCA extracts were tested with diphenylamine and their absorption spectra were determined on a Zeiss spectrophotometer. 0.25 mg. of highly polymerized DNA was used for the determination of the DNA spectrum.

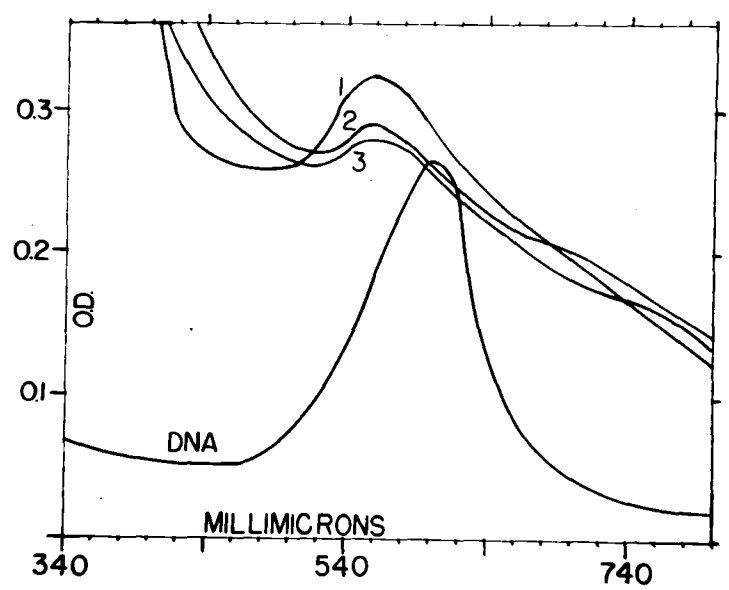

Figure XVI. Ultraviolet Absorption Spectra of the DPA Chromogen and of Highly Polymerized DNA.

Nuclei isolated from 20% of rat liver homogenates were precipitated with 5% TCA and washed once with TCA at 0°. The residues were resuspended in 10% NaCl solution and the pH was adjusted to 7.2. The resulting suspension mixtur was heated at 100° for 20 minutes. After centrifugation, the residues were resuspended in 10% NaCl solution and again heated at 100° for 20 minutes. After the second NaCl extraction, the residues were washed with water to remov all phenol red color and then extracted with 5% TCA at 90° for 15 minutes. 0.35 ml. of TCA extract (0.5 gm. tissue/ml.) was diluted to 3 ml. and read on Zeiss spectrophotometer against 0.35 ml. of TCA diluted to 3 ml. as a blank. 0.125 r of DNA dissolved in 3 ml. of water was read against water as a blank.

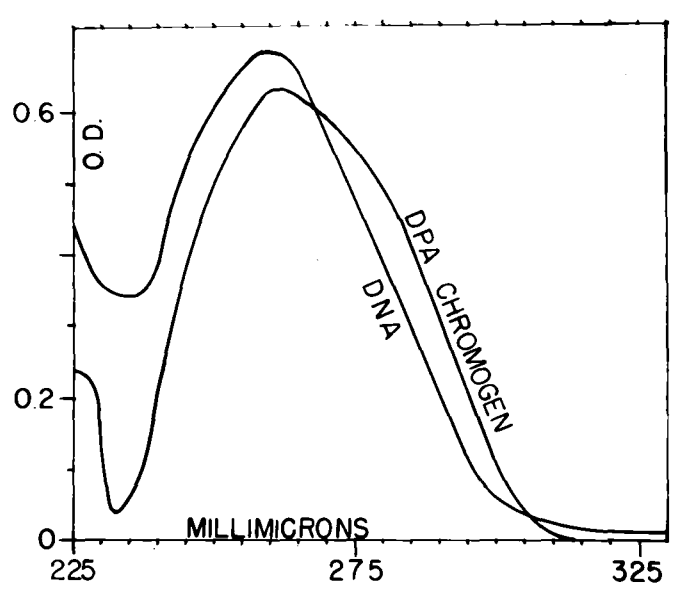

Figure XVII. Effect of DNA-ase on the Specific Activity of DNA Labelled by the Injection of Tritiated Thymidine into a Rat with a Regenerating Liver.

Two-thirds of the liver of an adult male rat was removed by partial hepatectomy and 10 microcuries of tritiated thymidine (0.360 curies/mM) was injected intraperitoneally 18 hours after the operation. The rat was sacrificed 5 hours later. The liver was removed, homogenized in a 0.25 M sucrose solution and the nuclei were isolated from 5 ml. aliquots by centrifugation at 600 x g. Nuclei were incubated with different concentrations of crystalline DNA-ase (indicated on the abscissa) using the standard incubation procedure (see text). The nuclei were then washed 5 times with 5% TCA at 0° and the DNA was extracted with 5% TCA at 90° for 15 minutes. The TCA extracts were extracted four times with 3 volumes of ether to remove all the TCA. The resulting aqueous solution was used for the determination of specific activity as c.p.m./mg. DNA (indicated on the left ordinate) and the determination of the content of DNA as mg. DNA/gm. tissue (given on the right ordinate). Every point at zero concentration of DNA-ase represents the average of duplicate determinations of the specific activities and DNA content.

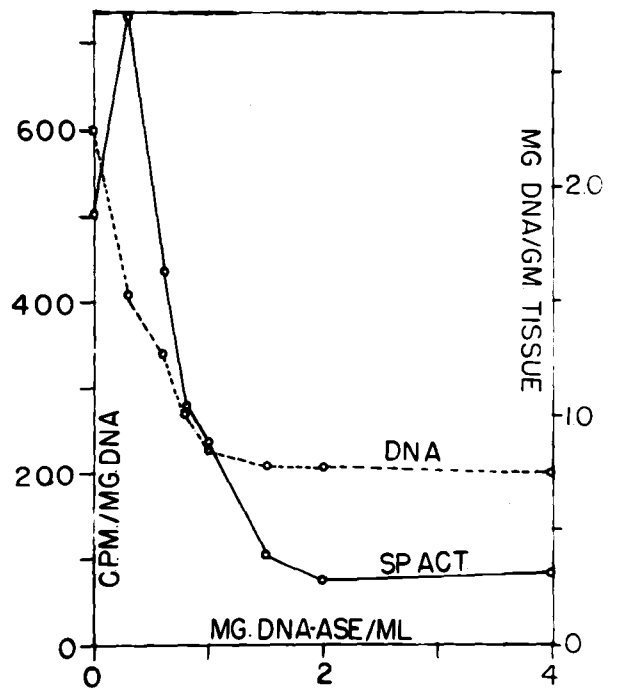

TABLE I

Effect of DNA-ase on the DNA Content and on the Specific Activities of Cytoplasmic and Nuclear RNA.

(See Legend for Figure X)

Exp. No.	DNA-ase (mg/ml)	DNA mg/gm tissue	% DNA removed	Specific Activity (counts/min/mg. RNA)			
				c-RNA	% inhibition	n-RNA	% inhibition
I	0	4.30	0	807	0	787	0
	2	1.04	76	500	38	682	14
	5	1.07	75	267	68	385	51
II	0	4.73	0	882	0	887	0
	3	1.58	67	534	40	723	18
	5	1.60	66	552	38	690	23
III	0	4.90	0	913	0	860	0
	2	1.50	70	541	40	758	12
	4	1.3	73	560	39	775	10

TABLE II

Effect of Removal of DNA on P^{32} Incorporation of Isolated Nuclei from Rabbit Appendix

Data by Sekiguchi and Sibatani (120)

Exp. No.	Concentration of DNA-ase (ug/ml.)	Amount of DNA (% of control)	total activity (counts/min./tube		
			OASP	RNA	DNA
M_{23}	0	100	13,950	1,160	137
	16	97	18,600	1,180	115
	63	92	19,000	782	83
	125	64	14,500	452	4
M_{21}	0	100	15,810	645	294
	125	31	15,010	150	21
	250	29	12,400	78	17
	500	27	9,500	0	18
	1,000	26	8,600	11	0

TABLE III

Effect of Manganese and Magnesium Ions on the Activity of Pancreatic DNA-ase

Nuclei isolated from 2.5 ml. aliquots of 20% rat liver homogenates were incubated with 1.5 mg./ml. of crystalline DNA-ase in solutions containing either 0.0075 M $MnSO_4$, 0.0045 M EDTA (ethylene diamine tetraacetic acid) and 0.06 M tris-acetate buffer (PH 8), or 0.04 M $MgCl_2$ and 0.02 M Phosphate buffer (PH 7), at 30° for 20 minutes. After the first 20 minutes of incubation, samples 7, 8, 11 and 12 were centrifuged, the supernatants were discarded and the nuclear pellets were reincubated with a fresh solution of DNA-ase (same conditions as first incubation) at 30° for 20 minutes. The remaining DNA and DPA chromogen were extracted with hot TCA and determined by the diphenylamine reaction.

Sample No.	Number of times incub. with DNA-ase	DNA-ase conc. mg./ml.	Ions Added	Mg. Apparent DNA/Gm. Tissue
1	1	0	Mn^{++}	4.04
2	1	0	Mn^{++}	3.98
3	1	0	Mg^{++}	4.01
4	1	0	Mg^{++}	3.94
5	1	1.5	Mg^{++}	1.02
6	1	1.5	Mg^{++}	0.96
7	2	1.5	Mg^{++}	0.92
8	2	1.5	Mg^{++}	1.02
9	1	1.5	Mn^{++}	1.65
10	1	1.5	Mn^{++}	1.82
11	2	1.5	Mn^{++}	1.08
12	2	1.5	Mn^{++}	1.08

TABLE IV

The Effect of Storing TCA Extracts of Rat Liver Nuclei at 0° C on the Content of DNA and DPA Chromogen

Nuclei were isolated from 2.5 ml. aliquots of 20% rat liver homogenate and the DNA was extracted from the nuclei by hot 5% TCA using the standard extraction procedure (see experimental). The TCA extracts were kept in ice for 22 hours, then centrifuged to remove any precipitate, and the supernatants were used for DNA determination by reaction with diphenylamine. Control samples were kept at room temperature. The absorption maxima were determined on a Zeiss spectrophotometer and found to be 600 millimicrons for all the samples.

Sample No.	Treatment	Mg. DNA/Gm. Tissue
I	Control	4.42
	Stored sample	4.40
II	Control	4.42
	Stored sample	4.42
III	Control	4.48
	Stored sample	4.48

TABLE V

Sugars Reacted with the DPA Reagent

Galactose	Arabinose
Sorbitol	Dulcitol
Levulose	beta-Glucose
Sorbose	Ribose
Mannose	Erythritol
Xylose	Lyxose
Raffinose	Adonitol
Rhamnose	Arabitol
Arabin	Fucose

TABLE VI

Effect of Alkaline Hydrolysis on the DPA Chromogen

Nuclei isolated from 2.5 ml. aliquots of 20% rat liver homogenates were washed twice with TCA at 0° C and extracted twice with 10% NaCl at 100° for 20 minutes. The residues were heated with 0.1 N and 0.2 N KOH at 80° for 10 minutes, the KOH was neutralized with 5% TCA at 0° and the solutions were centrifuged and the supernatants were discarded. The resulting residues were washed twice with cold TCA to remove any KOH, then extracted with 5% TCA at 90° for 15 minutes, and the DPA chromogen content was determined by reaction with diphenylamine.

Samples No.	Incubation solutions	Mg. of DPA Chromogen gm. tissue
1	0.1 N KOH	1.04
2	0.1 N KOH	0.92
3	0.2 N KOH	0.92
4	0.2 N KOH	0.94
5	Dist. H_2O	0.96
6	Dist. H_2O	0.92

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