Magnesium and Calcium in Isolated Cell Nuclei

H. NAORA, H. NAORA, A. E. MIRSKY, and V. G. ALLFREY

From The Rockefeller Institute. Dr. and Mrs. Naora' present address is The Cancer Institute, Tokyo

ABSTRACT The calcium and magnesium contents of thymus nuclei have been determined and the nuclear sites of attachment of these two elements have been studied. The nuclei used for these purposes were isolated in non-aqueous media and in sucrose solutions. Non-aqueous nuclei contain 0.024 per cent calcium and 0.115 per cent magnesium. Calcium and magnesium are held at different sites. The greater part of the magnesium is bound to DNA, probably to its phosphate groups. Evidence is presented that the magnesium atoms combined with the phosphate groups of DNA are also attached to mononucleotides. There is reason to believe that those DNA-phosphate groups to which magnesium is bound, less than $\frac{1}{10}$th of the total, are metabolically active, while those to which histones are attached seem to be inactive.

INTRODUCTION

The experiments to be described are concerned with the magnesium and calcium of the cell nucleus and with the significant differences in the biochemical distribution of such cations in the nucleus. The experiments indicate that magnesium is an important component in the formation of a complex structure in the nucleus; most of the magnesium is bound to DNA, some to RNA, and there is suggestive evidence that the magnesium bound to DNA is also shared by mononucleotides. Calcium is held in the nucleus in a different way.

The role of magnesium and calcium in the cell has progressively become more and more evident. These cations have been shown to be enzyme activators or to be essential metabolic factors in other ways (1). Magnesium especially plays a role in enzyme systems, especially in those concerned with phosphorylation and the synthesis of nucleic acids (2, 3) and proteins (4). It has been shown that some nucleoproteins are precipitated by very small amounts of calcium or magnesium (5, 6). Furthermore, these cations have an important role in maintaining the structure of cell components. Several observations have been made on this problem. In isolation of nuclei from thymus glands, magnesium or calcium must be added to the isolation medium (7). Experi-

ments employing the chelating agent (ethylenediaminetetraacetic acid, EDTA) have suggested the possible role of divalent cations in maintenance of chromosome integrity (8). It has also been suspected that the divalent cations are important in crossing over, since EDTA treatment increased the degree of crossing over in *Drosophila* (9) and *Chlamydomonas* (10). In the cytoplasm magnesium has been shown to be a factor responsible for the structure of ribonucleoprotein particles (11–13).

While problems concerning the biochemical importance of magnesium and calcium have led to a vast literature, almost nothing is known concerning the distribution and content of these cations in the nucleus. Thus it seemed desirable to investigate their distribution in the isolated nucleus.

1. *Nuclear Preparation and Content of Magnesium and Calcium in Isolated Nuclei*

1. MATERIALS TO BE USED

The ideal procedure to isolate nuclei for the purpose of the present investigation requires no loss and no adsorption of these cations during isolation. A number of procedures for isolating nuclei have been proposed for investigation on the metabolic activities or the fractionation of nuclear materials (13). A satisfactory preparation for the present work is the non-aqueous procedure of Behrens (41). In the present experiments a teflon homogenizer was used to disintegrate the frozen-dried tissue in order to prevent metal contamination.

We have also used nuclei isolated in sucrose solution from the thymus. The reason for working with these nuclei is that in a number of ways they are very active metabolically (7, 14). They can indeed be considered to be "surviving" nuclei. Non-aqueous nuclei, on the other hand, are inert metabolically. Labile calcium or magnesium linkages might very well be disrupted in non-aqueous nuclei. It was hoped that such linkages might be retained in sucrose nuclei and that some insight could be obtained into the role of calcium and magnesium in the metabolic activates of the nucleus.

The values for magnesium and calcium contents of non-aqueous nuclei have been taken to be the original contents of these cations in the cell nucleus and are used as a standard in considering the analyses of nuclei isolated in a sucrose medium. Such a standard is essential, for when a 0.25 M sucrose solution is used for nuclear isolation, it is usual to add either calcium or magnesium ions (0.003 M $CaCl_2$ or 0.003 M $MgCl_2$). The nuclei have the same appearance whether calcium or magnesium is used and in both cases they are metabolically active. Metabolic activity is lost if sucrose is removed.

2. ANALYTICAL RESULTS

Non-Aqueous Thymus Nuclei The calcium and magnesium contents of isolated non-aqueous nuclei are given in Table I. It can be seen that there

are no significant differences in magnesium concentration between non-aqueous nuclei and the corresponding "control" tissue (*i.e.*, tissue which has been lyophilized, ground, and exposed to the solvents used in the isolation of the nuclei); calcium was found to be slightly concentrated in nuclei.

The results obtained in this experiment indicate that, although nuclei isolated in sucrose require calcium or magnesium to maintain their morphological integrity (7, 17), nuclei do not selectively accumulate either calcium or magnesium *in vivo*.

TABLE I

AMOUNTS OF MAGNESIUM AND CALCIUM IN VARIOUS TISSUES AND NUCLEI

Animal and organ	Tissue or nuclei	Material* analyzed	Ca	Mg
			per cent	*per cent*
Mouse pancreas	Tissue	Dry	0.034	0.086
Calf kidney	Tissue	Dry	0.050	0.070
	Nuclei	2.3 M sucrose–0.001 M $CaCl_2$	0.533	0.061
Calf liver	Tissue	Fresh	0.005	0.016
		Dry	0.034	0.098
	Nuclei	2.3 M sucrose–no $CaCl_2$	0.043	0.163
		2.3 M sucrose–0.001 M $CaCl_2$	0.336	0.111
		0.6 per cent citric acid	0.002	—
Calf thymus	Tissue	Fresh	0.0035	0.021
		Dry	0.019	0.121
		Non-aqueous medium	0.015	0.114
	Nuclei	Non-aqueous medium	0.024	0.115
		0.25 M sucrose–0.003 M $CaCl_2$	0.594	0.082
		0.25 M sucrose–0.003 M $MgCl_2$	0.010	0.542
		0.6 per cent citric acid	0.000	0.005

* With the exception of the nuclei isolated in non-aqueous media, nuclear preparations were treated with ethanol and ether after isolation in their respective isolation media.

Williamson and Gulick (1944) in their work with non-aqueous nuclei reported a pronounced concentration of calcium and magnesium in nuclear fractions obtained from thymus tissue, lymph gland tissue, and human tonsils: calcium, 1.25 to 1.41 per cent; and magnesium, 0.07 to 0.10 per cent in nuclei; but calcium 0.73 per cent, and magnesium 0.02 per cent in the "whole thymus cell" (17). These figures are entirely different from ours. It may be that the high figures reported by Williamson and Gulick (17) include some contamination from the glass beads used in the prolonged milling of the tissue.

Sucrose Thymus Nuclei Cells of thymus glands were disrupted and nuclei were isolated in 0.25 M sucrose—0.003 M $CaCl_2$ (7, 16). The addition of calcium is necessary to prevent gel formation during isolation. From Table I, it can be seen that nuclei can combine with much calcium of the medium during isolation. As will be seen later, however, the extra calcium is bound at

different sites from those of the calcium naturally present in the nuclei. These nuclei contained more than 70 per cent of the magnesium content found in non-aqueous nuclei. The analyses of different preparations showed a variation (0.101 to 0.070 per cent) in magnesium concentration.

When 0.25 M sucrose–0.003 M $MgCl_2$ was used to isolate nuclei from thymus glands, magnesium of the isolation medium was found to be adsorbed by the nuclei. The calcium concentration of these nuclei is about half that of the non-aqueous nuclei. This finding shows that approximately 50 per cent of nuclear calcium is soluble, either not firmly bound to the nuclear materials, or bound to the materials soluble in the sucrose isolation medium.

It should be noted here that analysis of the bound magnesium in nuclei can be made with nuclei isolated in 0.25 M sucrose containing 0.003 M $CaCl_2$ and the tightly bound calcium with the nuclei isolated in sucrose containing 0.003 M $MgCl_2$. We shall refer to the nuclei isolated in sucrose containing $CaCl_2$ as "Ca-nuclei" and those isolated in sucrose containing $MgCl_2$ as "Mg-nuclei."

"Sucrose" Kidney and Liver Nuclei Clean calf kidney and liver nuclei can be isolated in 2.3 M sucrose containing 0.001 M $CaCl_2$. The procedure used was essentially that of Chauveau *et al.* (18), $CaCl_2$ being added to increase the yield. The calcium and magnesium contents of these nuclei are listed in Table I. As can be seen in this table, a large amount of calcium is adsorbed by nuclei when the sucrose solution used for isolation contains $CaCl_2$. The observed distribution patterns of calcium and magnesium showed a uniform distribution of these metals between nucleus and cytoplasm within a tissue, an exception being the values found for liver nuclei isolated in 2.3 M sucrose containing no $CaCl_2$.

It should be pointed out that liver nuclei isolated in 2.3 M sucrose are not metabolically active (19). There was presumably a change in composition or in structure of the nuclei during isolation which rendered these nuclei metabolically inactive and this change may have in some way been concerned with calcium or magnesium. Because hypertonic sucrose solutions inactivate nuclei (7) we have confined our attention to nuclei isolated under isotonic conditions. Because of their purity the thymus nuclei prepared in 0.25 M sucrose were selected for study.

Tissues The results of magnesium and calcium analyses of several tissues are summarized in Table I. Magnesium and calcium estimations were performed on both fresh and dry tissue. The lipid-free dry samples were prepared by treatment with alcohol and ether. Approximately 90 per cent of the magnesium and calcium content in the fresh thymus tissue was found in the dry samples. Such dry samples were used in the present experiments, unless otherwise indicated.

A slight variation of magnesium and calcium content was observed from sample to sample in each tissue. The results obtained with liver and kidney were in agreement with those hitherto reported (20). The concentration of magnesium was observed to be higher than that of calcium in mouse pancreas, calf kidney, liver, and thymus. The

highest ratio of magnesium to calcium was found in the thymus and the lowest in the kidney. But the measured values do not strictly indicate the concentration within cells, because extracellular fluids are known to contain both calcium and magnesium.

2. *Importance of Sucrose in Retention of Magnesium and Calcium in Ca-Nuclei and Mg-Nuclei*

Since the presence of sucrose in the medium is essential for maintaining the composition and metabolic activity of thymus nuclei, it was of interest to know whether maintenance of calcium and magnesium in isolated nuclei depends on the presence of sucrose. In testing the effect of removing sucrose from the medium, Ca- and Mg-nuclei were suspended in various media.

TABLE II

RETENTION OF MAGNESIUM AND CALCIUM IN NUCLEI PREPARED IN Ca-CONTAINING SUCROSE AND THEN SUSPENDED IN VARIOUS Ca-CONTAINING MEDIA*

Media	Retention	
	Mg	Ca
	per cent	*per cent*
0.25 M sucrose–0.003 M $CaCl_2$ (original suspension)	100	100
0.1 M tris buffer (pH 7.10)–0.003 M $CaCl_2$	2.6	105
0.25 M glycerol–0.003 M $CaCl_2$	0.7	149
0.25 M ethylene glycol–0.003 M $CaCl_2$	3.4	152

* Nuclear suspensions were prepared in the cold.

The results obtained are given in Table II. Almost all the magnesium was lost from Ca-nuclei when the sucrose medium (containing $CaCl_2$) was replaced with a tris buffer solution containing the same concentration of $CaCl_2$ at 1–2°C. Such a big loss of magnesium cannot be prevented by substitution of glycerol or ethylene glycol (neither of which media contained tris buffer) for sucrose, although solutions of these substances can be used to isolate nuclei, which have a satisfactory appearance when examined under the microscope (21, 22). (It has been shown that thymus nuclei isolated in isotonic glycerol or ethylene glycol have lost their capacity for amino acid uptake into proteins (13). These findings show that the presence of sucrose in the medium is essential to retain magnesium in Ca-nuclei. It should be noted at this point that there is a correlation between the retention of magnesium and of nuclear activities in Ca-nuclei, since 0.25 M sucrose is the necessary factor to maintain the nuclear activities *in vitro* (7); thymus nuclei isolated in glycerol or ethylene glycol look good but are metabolically inert (13).

In contrast to the magnesium of Ca-nuclei, the calcium content of Mg-

nuclei after removal of sucrose was found to be as much as 48.5 per cent of the amount in the original nuclei (Table III). As will be seen later, approximately one-half of the calcium content is easily released from Mg-nuclei in the presence of sucrose, but the rest of the calcium is very tightly bound to the nuclear materials.

TABLE III

RETENTION OF MAGNESIUM AND CALCIUM IN NUCLEI PREPARED IN MAGNESIUM-CONTAINING SUCROSE AND THEN SUSPENDED AT 1–2°C. OR AT 37°C. IN DIFFERENT MEDIA

No. of experiment	Condition	Media	Retention	
			Mg	Ca
			per cent	*per cent*
1	1–2°C.	0.25 M sucrose–0.003 M $MgCl_2$ (original suspension)	100	100
		0.1 M tris buffer (pH 7.07)–0.003 M $MgCl_2$	75.3	48.5
		0.1 M tris buffer (pH 7.09)–0.003 M $CaCl_2$	2.5	2520
2	37°C. for 30 min.	0.25 M sucrose–0.002 M $MgCl_2$–0.003 M phosphate buffer (pH 6.70)	12.4	56.4

3. *Retention of Magnesium and Calcium in the Isolated Nuclei in the Presence of Sucrose*

A series of experiments was carried out to determine what factors influence the release of magnesium from Ca-nuclei and of calcium from Mg-nuclei during incubation at 37°C. The purpose of these experiments was to find suitable conditions for later experiments dealing with the effect of DNAase and RNAase treatment on the release of calcium and magnesium from isolated nuclei.

The results obtained are presented in Tables III and IV. Almost all (85 to 94 per cent) the magnesium in Ca-nuclei can be maintained over a pH range 5.05 to 7.14 in various buffer solutions during 30 minutes' incubation at 37°C., as long as there is sucrose in the incubation medium. Approximately 10 per cent of magnesium is easily released from Ca-nuclei during incubation. Experiments showed a variation in the amount of the magnesium released from Ca-nuclei from one preparation to another. It can be assumed that a small portion of magnesium in Ca-nuclei is not firmly bound to the nuclear materials or is bound to soluble materials.

In contrast to the magnesium of Ca-nuclei, the calcium of Mg-nuclei seems to be easily released during incubation even if there is sucrose in the incubation

medium. As can be seen in Table III, the release of calcium from Mg-nuclei does not depend upon the presence of sucrose in the medium. One-half (49 to 56 per cent) of the calcium is released from Mg-nuclei either in the presence or the absence of sucrose and the rest of the calcium seems to be firmly bound to nuclear materials.

TABLE IV

RETENTION OF MAGNESIUM IN Ca-NUCLEI INCUBATED IN DIFFERENT SUCROSE-CONTAINING MEDIA

Media	Final concentration of buffer	pH	Mg retention
	M		per cent
No buffer (original suspension)	—	—	100
Phosphate buffer in 0.25 M sucrose	0.033	6.70	91.1
	0.033	7.14	87.1
Tris buffer in 0.25 M sucrose	0.033	5.05	88.7
	0.033	5.90	90.2
	0.033	6.72	89.2
	0.033	7.08	84.7
	0.067	7.10	94.1

Incubation: 30 minutes at 37°C.

4. *The Effect of DNAase on the Release of Magnesium and Calcium from Isolated Nuclei*

Experiments were performed to test whether DNAase affects the release of magnesium and calcium from non-aqueous nuclei and from sucrose nuclei—both Ca- and Mg-nuclei.

1. NON-AQUEOUS NUCLEI

Magnesium When non-aqueous nuclei were suspended in a pH 6.7 phosphate buffer in sucrose and incubated at 37°C. for 30 minutes *in the absence of DNAase*, only 1.5 per cent of DNA was lost from the nuclei and only 0.1 per cent of magnesium was released. Over 80 per cent of the mononucleotides were released from the nuclei. Under these conditions a gel formed.

When non-aqueous nuclei were treated with different concentrations of DNAase under the same conditions (without addition of either magnesium or calcium to the incubation medium), 5 to 56 per cent of the total DNA content was digested; and the DNAase treatment affected the release of magnesium from the nuclei. More than 75 per cent of the magnesium was lost from non-aqueous nuclei at the time DNA was digested by the enzyme. As can be seen in Fig. 1, there is a rapid release of magnesium from non-aqueous nuclei treated with DNAase when one-third of the DNA is removed and further re-

moval of DNA from non-aqueous nuclei did not show any marked release of magnesium under the conditions used. This experiment is an indication that magnesium is closely associated with DNA. One magnesium atom is released for nine DNA-P groups digested.

Calcium In contrast to magnesium, there was a considerable release of calcium from non-aqueous nuclei during incubation in the *absence* of DNAase. The DNAase treatment did not affect the further release of calcium from non-aqueous nuclei (Fig. 1).

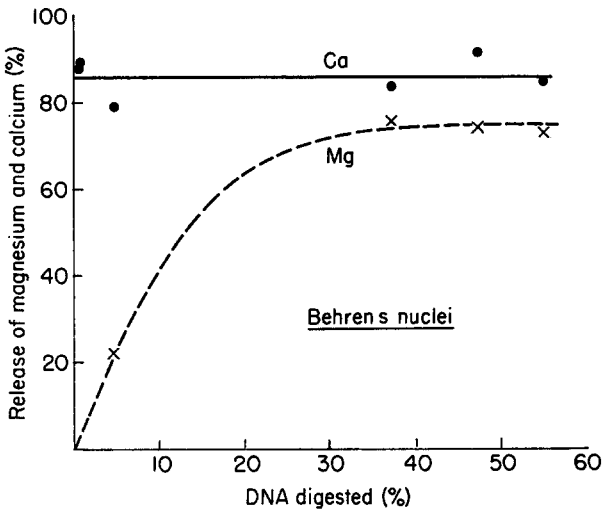

FIGURE 1. The effect of digesting DNA upon the release of native magnesium (x-----x) and of native calcium (●———●) from non-aqueous nuclei. Note that the calcium release is not dependent upon DNAase action.

2. SUCROSE NUCLEI

Magnesium It has already been noted that when sucrose nuclei are incubated in the absence of DNAase only a slight loss of magnesium occurs. When isolated Ca-nuclei were treated with different concentrations of crystalline DNAase in the *absence* of *added* magnesium in the incubation mixture (tris buffer in sucrose or phosphate buffer in sucrose), a maximum of 55 to 60 per cent of the total DNA content was digested (Fig. 2), although up to 84 per cent of DNA can be removed when magnesium is added to the incubation medium. The extensive digestion by DNAase which occurs without addition of magnesium is in itself an indication that some magnesium is already combined with DNA, for in experiments with isolated DNA no action of DNAase occurs in the absence of magnesium, and it is known that the activating effect of magnesium is due to combination with the substrate (23, 24). A correlation

between the DNA digested and the release of magnesium was found in either tris buffer–sucrose or phosphate buffer–sucrose media. The amount of magnesium released from Ca-nuclei on digestion of DNA is less than that from non-aqueous nuclei. One magnesium atom is released for 24 DNA-P groups released. This ratio of magnesium to DNA-P is less than was found for non-aqueous nuclei. The reason for this difference will be considered later.

The observed release of magnesium during DNAase treatment is directly related to the removal of DNA and not to the loss of RNA or naturally occurring mononucleotides. The determination of the RNA content of the DNAase-

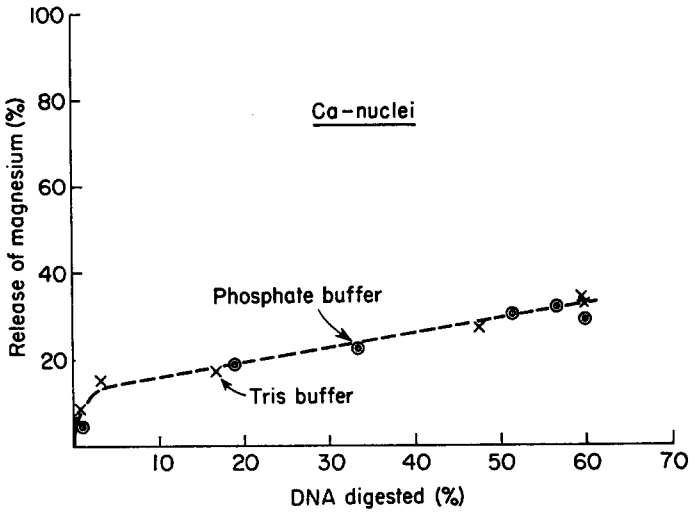

FIGURE 2. The effect of digesting DNA upon the release of native magnesium from nuclei isolated in 0.25 M sucrose–0.003 M $CaCl_2$. The nuclei were incubated in phosphate or tris buffer containing sucrose medium.

treated nuclei showed no release of RNA from the nuclei. It has previously been found that naturally occurring mononucleotides are not released from nuclei during DNAase treatment (15), or during incubation in the absence of DNAase. Although sucrose nuclei retain their mononucleotides under these conditions, non-aqueous nuclei lose 82 per cent of their mononucleotides simply on incubation (in the absence of DNAase).

The split products of DNAase digestion, mainly larger than trinucleotides (25), are not completely released into the medium during DNAase treatment. This material is removed from the nuclei by washing in a sucrose medium or by treatment with a strong acid. In a typical experiment 60 per cent of the DNA is digested and 37 per cent of the digested material is released without washing or acid treatment. The question arises as to how much magnesium remains in the nuclei attached to these split products. Unfortunately we have

not yet been able to obtain an unequivocal answer to this question because washing *after* incubation also tends to remove the naturally occurring mononucleotides.

Effects of DNAase Split Products and Histone upon Magnesium Retention

The results obtained in the present experiments indicate that magnesium is associated with DNA. The possibility must be considered, however, that the DNAase split products released are able to combine with the magnesium which had previously been bound to other nuclear materials and so carry off magnesium into the medium. A test for this possibility was carried out. The

TABLE V

EFFECT OF DENATURED DNA, SPLIT PRODUCTS OF DNA AND RNA DIGESTION, AND HISTONE UPON RETENTION OF MAGNESIUM IN Ca-NUCLEI*

Material added	Retention		
	DNA	RNA	Mg
	per cent	*per cent*	*per cent*
Control	100	100	100
Denatured DNA (5 mg.)‡	110	—	100
DNAase split products (8 mg.)	100	79.7	86.8
RNAase split products (2.9 mg.)	102	100	94.0
Histone I (5 mg.)	95.6	93.1	98.7

* Nuclei were incubated in 1.5 ml. of the mixture containing 0.25 M sucrose, 0.033 M tris buffer (pH 6.70–6.74), 0.002 M $CaCl_2$, and the listed materials, for 30 minutes at 37°C.
‡ Phosphate buffer–sucrose–$CaCl_2$ (pH 6.70) was used.

first step was to prepare the split products formed by DNAase digestion. This was done as follows:—

100 mg. of thymus DNA were hydrolyzed by 3 mg. of DNAase in the presence of 0.003 M $MnCl_2$ at pH 6.8. DNAase split products of the DNA digestion were dialyzed against 250 ml. of double distilled water in the cold. The material that passed through the membrane was condensed and then shaken five times with 2 volumes of 5 per cent 8-quinolinol in chloroform to remove metals. Finally this solution was shaken with chloroform and ether, five times each. Neither magnesium nor manganese was detected in the DNAase split products obtained. When Ca-nuclei were incubated in a medium containing the DNAase split products for 30 minutes at 37°C., the nuclei lost only a very small amount of their magnesium as can be seen in Table V. The amount of the added split products was 70 per cent as much as the total DNA content of the nuclei suspended in the tube. RNA was also observed to be released from

the nuclei under this incubation condition. It is not quite clear, therefore, whether the slight release of magnesium is (*a*) due to the RNA release from nuclei, since magnesium is also associated with RNA or (*b*) due to chelation by the DNAase split products used.

Another test for chelation by the DNAase split products was carried out. It has been observed that, at a low concentration of sodium chloride, magnesium is tightly bound by both undenatured and denatured DNA (26, 27). Therefore, a test was performed as to whether the magnesium bound to isolated DNA could be removed by DNAase split products. To 12.5 ml. of a solution in 0.002 M NaCl of DNA, containing 0.0093 mg. DNA-P per ml. was added 0.6 to 0.7 equivalent of magnesium per DNA-P and 0.16 ml. of a solution of DNAase split products, containing an amount of material equivalent to the quantity of DNA present. The DNA was separated from the DNAase split products by centrifugation at 40,000 R.P.M. for 20 hours. The magnesium analyses of the supernates which contained the added split products, showed no difference in magnesium content between the control (Mg + DNA) and experiment (Mg + DNA + DNAase split products). This result shows that the magnesium bound to DNA was not removed by the added split products.

Therefore, it seems unlikely that during the release of DNAase split products from the DNAase-treated nuclei, these products took off the magnesium which had been bound to nuclear materials.

It is conceivable now that the slight amount of magnesium released from the nuclei during incubation in the presence of DNAase split products is due to the RNA release which occurs in some way and that such RNA release is perhaps prevented by the free histone in the DNAase-treated nuclei, since some histone has been observed to be released from the nuclei in the course of DNAase treatment. The next question to be considered, therefore, is whether histone combines with magnesium during DNAase treatment. When Ca-nuclei were incubated in the presence of histone (5 mg. thymus arginine-rich histone per 1.5 ml. incubation medium), nuclear magnesium was well retained in the nuclei (Table V). There are therefore no grounds for supposing that magnesium released when DNA is digested is due to the removal of magnesium by the released histone.

(It should be mentioned in passing that substitution experiments (40) have made it clear that large molecules like DNA and histone can enter the nucleus. Therefore the above experiments are not subject to the limitation that the added compounds may not have been able to penetrate the nuclear membrane.)

Calcium Experiments were done to test the effect of DNAase on the release of calcium from Mg-nuclei. First, however, Mg-nuclei were incubated in the absence of DNAase, and it was found that 43 per cent of their calcium

was immediately released. No further release of calcium was found on subsequent treatment with DNAase, as shown in Fig. 3. This is in accord with the results obtained using non-aqueous nuclei. There is no evidence, therefore, that calcium is bound to DNA.

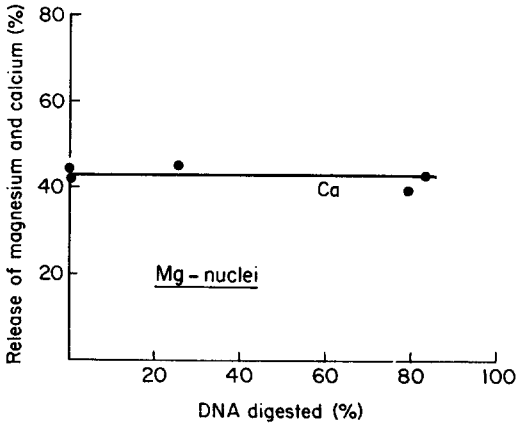

FIGURE 3. The effect of digesting DNA upon the release of native calcium from nuclei isolated in 0.25 M sucrose–0.003 M $MgCl_2$.

5. *The Effect of Removal of Nucleotides on the Magnesium and Calcium of Ca- and Mg-Nuclei*

Magnesium As described in this report, the magnesium retention in isolated nuclei is entirely dependent upon the presence of sucrose. As soon as sucrose is replaced by a buffer in water or by glycerol and ethylene glycol at the same molar concentration as the sucrose, the nuclei lose their metabolic activities and also their magnesium. It was found previously that mononucleotide retention in the nuclei is also dependent upon the presence of sucrose (28). It is quite possible, therefore, that the loss of magnesium when sucrose is removed is related to the release of mononucleotides.

There is further evidence suggesting a correlation between the release of ultraviolet-absorbing materials (presumably nucleotides) and of magnesium from isolated nuclei. When Ca-nuclei are washed once, twice, and three times with incubation medium in the cold *after incubation* as usual, the nuclei lose ultraviolet-absorbing materials (cold PCA-soluble) and magnesium at each step of washing. The quantity of material released at each washing into the medium and which remained in solution after addition of perchloric acid (final concentration 2 per cent) was assayed by its absorption at 260 mμ. A certain quantity of such material is also released from nuclei by treatment with cold 2 per cent perchloric acid. At each washing the loss of material extractable from nuclei with perchloric acid was equal to that found in the washings.

After each washing the magnesium content of the nuclei was determined, as well as their contents of DNA and RNA. There was an excellent correlation between the loss of magnesium and the loss of material soluble in perchloric acid and absorbing at 260 mμ. Thus at the end of three washings 42 per cent of the magnesium and 42 per cent of the ultraviolet-absorbing material were lost. At the same time there was no loss of DNA and a slight loss (15 per cent) of RNA.

Further experiments demonstrate a more specific relationship between the release of magnesium and of mononucleotides. It has been observed that when Ca-nuclei are treated in the cold with a dilute acetate buffer in sucrose, of which the pH is below 5.1, mononucleotides are immediately released from nuclei. This effect was found to be dependent upon the pH and also on the chemical composition of the buffer solution used for it occurs only in acetate buffers, not in other buffers, succinate for example, at the same pH and ionic strength (28). It was confirmed in the present experiment using the technique of column adsorption on Dowex 1 (formate) that an acetate buffer in sucrose at low pH values selectively extracts mononucleotides from Ca-nuclei.

To study the relationship between the removal of mononucleotides and the release of magnesium from Ca-nuclei, extractions were carried out in two buffer systems at two pH values, 4 and 6. In one series, 0.5 ml. (about 30 mg., dry weight) of nuclear suspension was added to 4.5 ml. of 0.022 M *acetate* buffers in 0.25 M sucrose–0.003 M $CaCl_2$, pH's 4 and 6. Such mixtures were kept at 1–2°C. for 30 minutes. In a second series succinate-sucrose buffers of the same molar concentration and pH values were used. These tubes served as "controls" because succinate buffers do not selectively release mononucleotides from the nucleus.

It is evident from the results obtained (Table VI) that there is a correlation between the retention of mononucleotides and of magnesium in Ca-nuclei. Although the values obtained on the retention of mononucleotides and of magnesium in the Ca-nuclei treated with acetate buffer in sucrose showed a variation in each experiment, it was found that the amount of the released magnesium was almost proportional to that of the lost mononucleotides. Assuming an average molecular weight for mononucleotides of 350, calculation shows that the ratio in sucrose nuclei is one mononucleotide to one or two magnesium atoms.

Our experiments indicate that the bulk of the bound magnesium is closely associated with mononucleotides in the nuclei. Such evidence is supported by experiments showing complex formation between ADP and ATP and a divalent cation (29). At this point a reference should be made to non-aqueous nuclei. It was mentioned above that when these nuclei are incubated, 82 per cent of their mononucleotides are lost at once and yet practically all their magnesium is retained. This behavior differs from that observed in the meta-

bolically active sucrose nuclei. Since mononucleotides in sucrose nuclei are held in a highly labile condition it is not surprising that the structure of the complex is affected by lyophilization and subsequent treatment with organic solvents.

TABLE VI

EFFECT OF ACETATE BUFFER ON RETENTION OF MAGNESIUM IN Ca-NUCLEI

Media*	pH	Retention	
		Nucleotides (E_{260})	Mg
		per cent	*per cent*
Original nuclei (no buffer)	—	100	100
Acetate buffer	4.21	15.6	30.3
	6.01	69.6	76.5
Succinate buffer	4.02	63.6	80.0
	6.01	72.6	71.1

* Final concentration of the medium: 0.02 M buffer solution, 0.003 M $CaCl_2$, 0.25 M sucrose

TABLE VII

EFFECT OF NUCLEOTIDES ON RETENTION OF MAGNESIUM IN Ca-NUCLEI

Material added	Retention of magnesium
	per cent
Control (no nucleotide)	100
AMP* (4.5 mg.)	101
ADP‡ (5.5 mg.)	90.7
ATP‡ (6.5 mg.)	89.4

* Nuclei were incubated in AMP containing 0.033 M phosphate buffer–sucrose (pH 6.70) for 30 minutes at 37°C.

‡ Nuclei were suspended in ADP or ATP containing 0.02 M acetate buffer–sucrose (pH 6.60) for 30 minutes at 1–2°C.

Effects of Nucleotides upon Magnesium Retention The possibility must be considered that during the release of mononucleotides by treatment with an acetate buffer solution, the mononucleotides carried off magnesium which had had been bound to other nuclear materials. The following experiment was therefore done. Ca-nuclei (36 to 47 mg. in dry weight) were suspended in the presence of an excess of adenosine-5′-diphosphate or triphosphate in the cold, or incubated in the presence of adenosine-5′-monophosphate. The amounts of the mononucleotides used are listed in Table VII. It was found that the amount of mononucleotides in 50 mg. of Ca-nuclei was about 0.6 mg. (28). Therefore the amount of each mononucleotide used in this experiment was far

greater than the amount of mononucleotides naturally occurring in the nuclei. AMP had no effect on magnesium release. ADP and ATP were found to cause a slight release of magnesium (9 to 11 per cent). Considering the large amount of the added mononucleotides, the observed effect indicates that mononucleotides when released from nuclei do not remove a significant amount of magnesium bound at other sites within the nuclei. This conclusion was confirmed by experiments on isolated DNA. To undenatured DNA in aqueous solution enough magnesium was added to form a Mg–DNA complex containing the maximum amount of magnesium. An excess of ATP was added to the solution

TABLE VIII
EFFECT OF ACETATE BUFFER ON RETENTION OF CALCIUM IN Mg-NUCLEI

Media*	pH	Retention	
		Nucleotides (E_{260})	Ca
		per cent	*per cent*
Original nuclei (no buffer)	—	100	100
Acetate buffer	4.00	19.8	23.8
	6.00	83.2	63.3
Succinate buffer	3.78	81.8	25.9
	6.05	83.2	43.3

* Final concentration of the medium: 0.02 M buffer solution, 0.003 M $MgCl_2$, 0.25 M sucrose.

which was then centrifuged at high speed to sediment the DNA. A magnesium determination on the sediment showed that no magnesium was removed from DNA by ATP.

Calcium Mg-nuclei were used to find the effect of acetate buffer in sucrose on the release of calcium. In Mg-nuclei as in Ca-nuclei, acetate buffers in a certain pH range released mononucleotides. The results obtained are listed in Table VIII. An acetate buffer in sucrose at pH 4.00 releases calcium from Mg-nuclei. This, however, is not a specific effect of acetate buffer, for when Mg-nuclei were suspended in a succinate buffer in sucrose at low pH, calcium was also released, and yet in this case there was no release of mononucleotides. There is, therefore, no evidence that calcium is associated with mononucleotides. Once again, calcium and magnesium are found to be held at different sites.

6. *The Effect of RNAase on the Release of Magnesium from Ca-Nuclei and of Calcium from Mg-Nuclei*

When Ca-nuclei are incubated in the presence of RNAase at various concentrations (1 to 1000 μg./1.5 ml. incubation medium) for 30 minutes at 37°C.,

different amounts of RNA are digested. It was found, as can be seen in Fig. 4 *a*, that the digestion of RNA by RNAase under our conditions is not proportional to the concentration of enzyme used. It should be mentioned that during incubation of Ca-nuclei in the absence of RNAase, 16 per cent (average) of their RNA is released. This is referred to as the medium-soluble RNA.

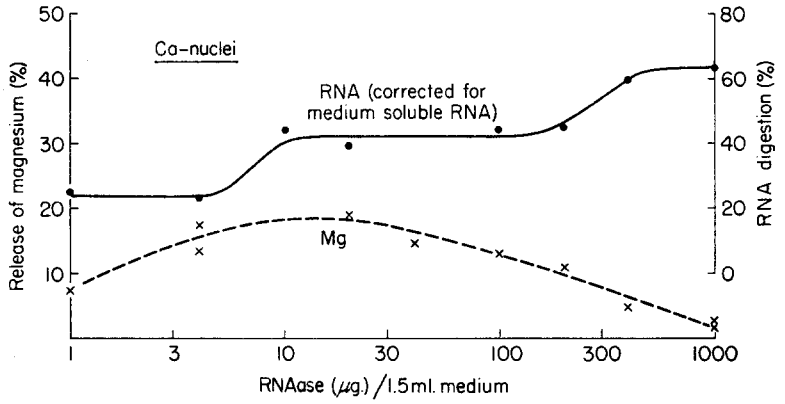

FIGURE 4 *a*. The effect of RNAase on the RNA content and on the release of magnesium from nuclei isolated in 0.25 M sucrose–0.003 M $CaCl_2$.

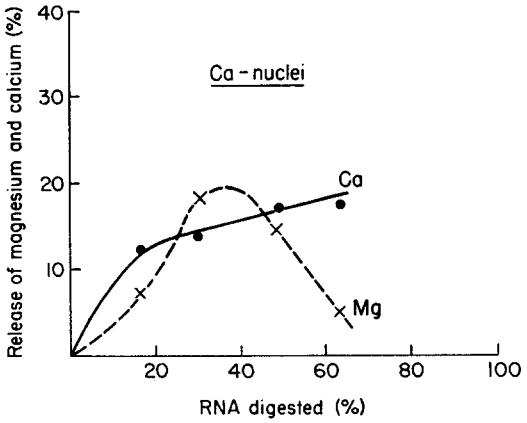

FIGURE 4 *b*. The effect of digesting RNA upon the release of native magnesium (x-----x) and artifact calcium (●———●) from nuclei isolated in 0.25 M sucrose–0.003 M $CaCl_2$.

Magnesium To test whether RNAase affects the release of magnesium from Ca-nuclei, the concentration of RNAase in the incubation medium was varied. The results are given in Figs. 4*a* and *b* which show the relation between the release of magnesium and RNA from Ca-nuclei. The highest values for the release of magnesium are observed for about 40 per cent digestion of RNA. When more enzyme is used and more RNA is digested, the amount of

magnesium released declines. It seems likely that the decrease in magnesium release is due to the formation of a magnesium–RNAase complex, which remains in the nuclei. The fact that magnesium ions inhibit the activity of RNAase (12) is in line with the suggestion that magnesium forms a complex with the enzyme.

After removal of the RNAase-containing incubation medium by centrifugation, some of the hydrolyzed RNA still remained in the nuclei just as DNAase split products remained in the nuclei after DNAase treatment. When the RNA split products were removed by washing with the incubation medium twice in the cold, more magnesium was found to be released from nuclei, but the results are difficult to interpret because at the same time some of the naturally occurring mononucleotides are released.

Effects of RNAase Split Products upon Magnesium Retention It is possible that, during release of the digested RNA from nuclei, the magnesium which had been associated with other nuclear materials was carried off by the split products of RNA digestion. The same possibility was considered for the release of magnesium accompanying DNAase digestion. An experiment was performed to test such a possibility. Yeast RNA was washed with 10^{-1} M versene (at pH 7.0) five times by the procedure of Wacker (30) and then dialyzed against a large volume of distilled water to remove the versene used. Then 200 mg. of the washed RNA were hydrolyzed with 5 mg. RNAase for 1 hour at 37°C. The digested material was dialyzed in the cold against double distilled water and the material which passed through the membrane was collected. It was observed by Wacker and Vallee (30) that beef liver RNA treated with versene six times still contained a small amount of magnesium and other metals. But, the contamination of magnesium in the split products which were obtained in our experiment could be completely neglected. Ca-nuclei were incubated in the presence of 2.9 mg. of the dialyzable split products. Such an amount of the split products used in this experiment was 3.2 times higher than the total RNA content of the original nuclei and more than 8 times higher than the amount of the RNA released by the treatment of the enzyme in the experiments showing the effect of RNAase on the release of magnesium. The result obtained is shown in Table V in which the magnesium retention of nuclei after incubation in the presence of the split products is compared with that of the control nuclei. No significant effect on the release of magnesium from Ca-nuclei was observed.

Accordingly, it may be concluded that the magnesium released by RNAase is closely associated with RNA in the cell nucleus. There are reports, it should be noted, that isolated RNA can bind magnesium (30, 31).

Calcium Experiments were done to test the effect of RNAase on the release of calcium from Mg-nuclei. In such nuclei there was almost no effect of

RNAase. This is probably due to the inhibition of RNAase by magnesium ions (12). During incubation 44 per cent of their calcium was released from Mg-nuclei whether or not RNAase was present.

7. *Differences between "Native" and "Artifact" Magnesium and Calcium in Isolated Thymus Nuclei*

As noted previously, Ca-nuclei were isolated in 0.25 M sucrose–0.003 M $CaCl_2$ and Mg-nuclei in 0.25 M sucrose–0.003 M $MgCl_2$. Calcium or magnesium ions were added to prevent nuclear gel formation. It was shown that nuclei adsorbed these cations from the isolation medium. Of particular interest was the observation that such adsorbed magnesium or calcium behaves differently from the cations originally present in the cell nucleus. We shall refer to the former cations as "artifact" and to the latter ones as "native."

The native calcium content of thymus nuclei, obtained by analyses on non-aqueous nuclei, is 0.024 per cent whereas the artifact calcium content is 0.594 per cent. The native magnesium content, from analyses on non-aqueous nuclei, is 0.115 per cent and the artifact magnesium content is 0.542 per cent.

Magnesium It was noted previously in connection with the effect of sucrose on magnesium retention that native magnesium is not readily released from nuclei during incubation (when no enzyme is added). On the other hand, 88 per cent of artifact magnesium is rapidly released during incubation and so the bulk of the artifact magnesium appears to be bound at a different site from that occupied by native magnesium.

A part, however, of the extra magnesium in Mg-nuclei is held in somewhat the same way as is native magnesium; *i.e.*, in combination with DNA. This is shown by the fact that when magnesium is added to a nuclear suspension more DNA is digested than when no magnesium is added—63 to 84 per cent of the total DNA being digested when magnesium is added and 50 to 60 per cent when no magnesium is added. Since the activating magnesium is known to combine with the substrate, the increase in amount of DNA digested is evidence that some of the added magnesium combines with DNA. Measurements on magnesium released by action of DNAase from nuclei to which magnesium has been added (and the measurements are made after removal of the bulk of the added magnesium by a preliminary incubation in the absence of DNAase) show that the extra amount of magnesium released ("extra" meaning the amount greater than that released from nuclei to which no magnesium was added) is about one-tenth of the total native magnesium.

This experiment shows that even when a great excess of magnesium is added

to sucrose nuclei, a large amount of magnesium is adsorbed by the nuclei but only very little combines with their DNA. The reason for this is that, as will be shown later, magnesium combines mainly with the phosphate groups of DNA and these groups in nuclei are already combined with histones.

Calcium Much of the native calcium is released during incubation even when no enzyme is added and that which remains is not released by DNAase. Artifact calcium, on the other hand, is firmly held during incubation when no

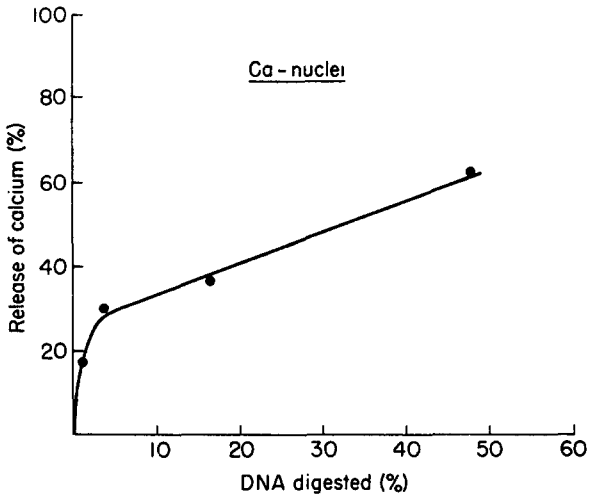

FIGURE 5. The effect of digesting DNA upon the release of artifact calcium from nuclei isolated in 0.25 M sucrose–0.003 M $CaCl_2$.

enzyme is added; and when either DNAase or RNAase is added, artifact calcium is released, about 60 per cent by DNAase (Fig. 5) and 20 per cent by RNAase. The conclusion is that native and artifact calcium are held at different sites.

8. *The Combination of Magnesium with Isolated DNA*

Ten samples of undenatured DNA, each containing 0.113 mg. of P, were dissolved in dilute NaCl solutions, five of which were 0.002 M and the other five 0.02 M. To each solution varying amounts, up to 1.92 equivalents, of magnesium per DNA-P were added. The solutions were centrifuged in the Spinco at 40,000 R.P.M. for 20 hours. After centrifugation, supernates were carefully separated from sediments. The amounts of DNA sedimented were measured by absorption at 260 mμ and by the diphenylamine reaction. The amounts of

magnesium remaining in the supernates and precipitated with DNA were measured. From these data the amounts of magnesium bound to DNA were calculated (Fig. 6).

This experiment is discussed in relation to previous work on the combination of DNA with magnesium in the next section of this paper.

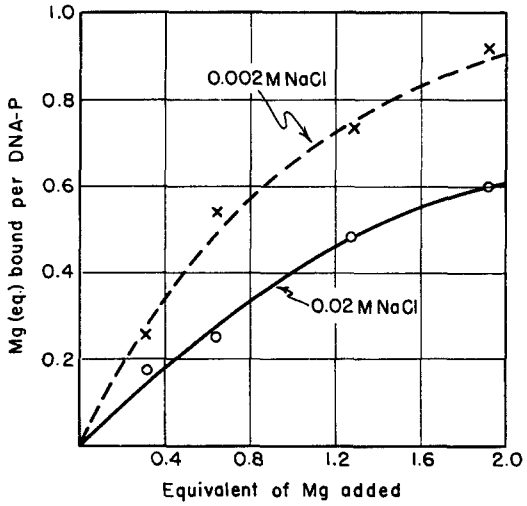

FIGURE 6. Magnesium-binding capacity of undenatured DNA dissolved in 0.002 M (x-----x) and 0.02 M (o———o) NaCl solution.

9. *Biochemical Distribution of Magnesium and Calcium in Cell Nuclei*

The experiments described demonstrate two different characteristics of the biochemical distribution of divalent cations in isolated thymus nuclei. One of these, which has been discussed in section 7, is the difference between native and artifact magnesium and calcium. The other characteristic concerns the differences in the binding sites for native magnesium and calcium. These differences have been mentioned throughout the paper. The data are summarized in Fig. 7. The contents of magnesium and calcium in non-aqueous thymus nuclei were taken as giving the over-all amounts present in those nuclei.

Calcium and magnesium have such different properties in biological systems that it is hardly surprising that they have different distributions within the nucleus. And yet previous work on calcium and magnesium in the nucleus has not been concerned with the differences in distribution of the two elements within the nucleus. Thus, the presence of a conspicuous amount of ash (much of it probably due to calcium and magnesium) in nuclei was observed by the

classical histochemical techniques (32–35). More recently there have been cytological studies using chelating agents which suggested that magnesium *and/or* calcium may be required to maintain chromosome structure (9). In a recent study in which separate determinations of calcium and magnesium were made (36) the so called "nuclear fraction" of liver homogenate was used, but unfortunately such a nuclear preparation is not suitable for satisfactory analyses (13).

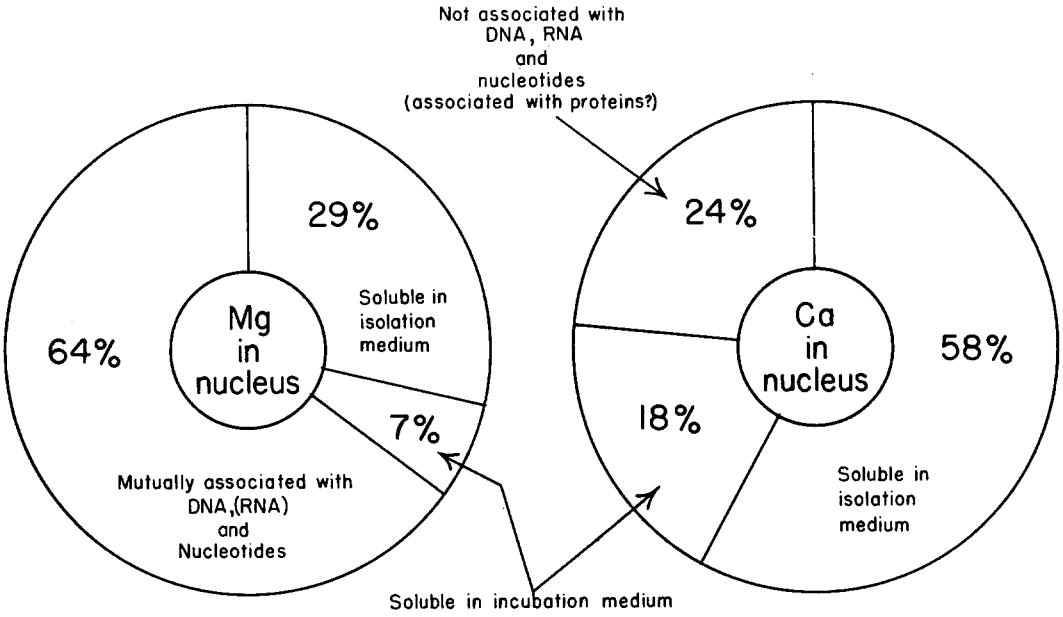

FIGURE 7. A schematic drawing of the biochemical distribution of native magnesium and calcium in the thymus cell nucleus. Note the difference between tightly bound magnesium (64 per cent) and calcium (24 per cent).

The Distribution of Calcium The experiments described in this paper indicate that the firmly bound calcium of the nucleus (which is 24 per cent of the total nuclear calcium) is not associated with either DNA, RNA, or mononucleotides, since no particular correlation between calcium release and the removal of these nuclear materials was observed. Although the loosely held calcium may be attached to nucleic acids, it is likely that the firmly bound calcium is attached to protein. In this connection an interesting observation on calcium in the nucleus by Steffensen and Bergeron (37) should be mentioned. Pollen of the Easter lily labeled with Ca^{45} was allowed to develop into pollen tubes. All the nuclei, namely the tube nucleus and the two sperm nuclei, were observed autoradiographically to retain Ca^{45}. This means that calcium was retained after a mitotic division and it was therefore inferred that

calcium was bound in a stable linkage with a continuing component of the chromosomes, possibly the DNA. Our experiments suggest that the retained Ca^{45} might have been attached to a chromosomal protein.

The Distribution of Magnesium It can be seen from Fig. 7 that 64 per cent of total nuclear magnesium is closely associated with DNA, RNA, and mononucleotides. The release of firmly bound magnesium from the isolated nuclei is observed to follow the removal of either DNA, RNA, or mononucleotides; and unless mononucleotides, RNA, and DNA are removed from Ca-nuclei, the bound magnesium is well retained. The correlation between magnesium release and the removal of DNA, RNA, and mononucleotides was not due to transfer of the magnesium from combination with other nuclear materials to split products formed by the digestion of DNA or RNA or released mononucleotides. All the evidence therefore indicates that magnesium is combined with these components, but it must be said that the evidence falls far short of proof.

Since the sum of the amounts of magnesium removed with each of the individual components—DNA, RNA, and mononucleotides—is greater than the total magnesium content of the nucleus, we would suppose that the magnesium is held in mutual complex by all three or at least by two of them—DNA and mononucleotides. This would mean that in the nucleus mononucleotides are linked by magnesium atoms to DNA. In the thymus nucleus there are between one and two atoms of magnesium for each mononucleotide. Under some conditions (non-aqueous nuclei) the mononucleotides are released from nuclei, leaving all the magnesium combined with DNA: under other conditions (removal of sucrose from a preparation of sucrose nuclei or action of acetate on sucrose nuclei) release of mononucleotides from the nuclei is accompanied by a massive release of magnesium, although all the DNA remains in the nuclei; and under still other conditions (release of DNA by DNAase from sucrose nuclei) part of the magnesium is released along with the DNA. The sharing of magnesium by DNA and mononucleotides would explain these observations. To which of these two components the magnesium remains attached when one of them is released from the nucleus depends upon factors which we do not yet understand.

The nature of this hypothetical linkage between mononucleotides and DNA will be better understood if we consider what is known about how the mononucleotides are held in the nucleus (28) and how magnesium is bound by DNA. The nucleotides found in isolated thymus nuclei are diphosphopyridyl nucleotide, uridylic acid, cytidylic acid, guanylic acid, and adenylic acid, of which the last is present in by far the highest concentration. Phosphorylation of AMP by an oxidative process readily occurs in isolated thymus sucrose nuclei, but only the AMP already present in the nucleus is phosphorylated;

added AMP remains unchanged. This suggests that the AMP of the nucleus occupies a special site. Further evidence for this comes from the "acetate effect." When acetate in low concentration is added to a suspension of sucrose nuclei at 0°C. the mononucleotides are immediately released.

The interaction of magnesium and DNA has been repeatedly studied in recent years. According to Zubay and Doty (38, 39) undenatured DNA binds magnesium weakly, while heat-denatured DNA binds it more strongly. The site of binding was claimed to be mainly at adenine and guanine. This work has been criticized by Felsenfeld and Huang (27) and by Shack and Bynum (26). Their experiments demonstrated that magnesium is tightly bound to either undenatured or heat-denatured DNA and that the binding of magnesium by DNA involves primarily the charged phosphates rather than amino or enolic groups of purines. Evidence that binding of magnesium by DNA occurs at phosphate groups was the observation that binding is blocked by increasing the concentration of sodium chloride. At a very low concentration of sodium chloride, 0.002 M, there is, according to Shack and Bynum, 0.6 equivalent of magnesium per nucleotide of DNA.

We have measured the combination of magnesium with DNA by a method that is more direct than that previously used. Magnesium was added to a DNA solution which was then centrifuged at high speed, so that well over 90 per cent of the DNA was sedimented. The quantity of magnesium combined was found by analyzing the sediment or determining the amount of magnesium removed from the supernate. Undenatured DNA in 0.002 M sodium chloride combined with 0.9 equivalent of magnesium per DNA-P which approximates one magnesium atom to two nucleotides. The blocking effect of higher concentrations of sodium chloride was confirmed. The details of this experiment are given in section 8 of this paper.

Putting together what is known about the site of attachment of magnesium to isolated DNA, the manner in which magnesium is held in the nucleus, and the complex of which mononucleotides seem to form a part, it may be postulated that in the nucleus, phosphate groups of DNA are linked by magnesium atoms to mononucleotides.

Magnesium and the Active Sites of Chromosomes If it be assumed that nearly all the magnesium which is tightly bound in thymus sucrose nuclei is held by DNA we find that this is somewhat less than one-tenth of the maximum combining capacity of the amount of undenatured DNA present. Furthermore, as has been mentioned in this paper, if a great excess of magnesium chloride is added to sucrose nuclei, although much magnesium becomes rather loosely attached, very little (not more than 10 per cent of the quantity already present) is bound to DNA. There is something that prevents more than one-tenth of the phosphate groups of the DNA within a nucleus from combining with

magnesium. It is known that in the nucleus the phosphate groups of DNA are combined primarily with histones. Apparently this combination tends to prevent the phosphate groups of DNA from combining with magnesium just as it prevents them from combining with basic dyes. Experiments which we have done with isolated undenatured DNA dissolved in 0.002 M NaCl show that the presence of arginine-rich histone does in fact block the combination of magnesium with DNA.

In a chromosome, then, some patches of DNA phosphate groups are attached to magnesium and thus, perhaps, to mononucleotides; while the rest of the phosphate groups are blocked by histone. Experiments on isolated sucrose nuclei have clearly demonstrated that the phosphate groups of DNA are sites of activity, for activity is retained when DNA is replaced by polyanions such as polyethylene sulfonate or polyacrylic acid (40). The negative electrical charge of the phosphate group is all that phosphate has in common with the polyanions that can effectively substitute for it. Polyacrylamide, lacking a negative charge, is ineffective as a substitute for DNA that has been removed. Nuclei which still possess their DNA lose much of their activity when polycations such as polylysine or histone are added to them.

An essential nuclear activity which is made possible by the phosphate groups of DNA is phosphorylation of mononucleotides, the formation of ATP. The magnesium atoms which are combined with phosphate groups are at sites of activity, possibly linking together active patches of the long DNA chains with the mononucleotides.

EXPERIMENTAL

1. Materials Calf kidney, liver, and thymus were transported to the laboratory in ice cold 0.25 M sucrose as quickly as possible after the death of the animal. Fat-free, dry mouse pancreas stored at −20°C. was used. The pancreas had been lyophilized and then extracted with petroleum ether.

2. Solutions and Apparatus Solutions were made with double distilled water. Caution was taken to prevent contamination from air-dust. Glass apparatus was cleaned with sulfuric acid cleaning solution and then rinsed with a large volume of deionized water and again with double distilled water.

3. Preparation of Thymus Nuclei Ca-nuclei were prepared by the procedure of Allfrey *et al.* (7). Caution was taken to prevent contamination by magnesium during isolation. The nuclei were finally suspended in 0.25 M sucrose–0.003 M $CaCl_2$, 1 ml. containing 30 to 50 mg. of nuclei. Mg-nuclei were prepared with 0.0033 M and 0.003 M $MgCl_2$–0.25 M sucrose. The procedure for preparing these nuclei was otherwise the same as that for Ca-nuclei. 1 ml. of Mg-nuclear suspension usually contained 50 to 60 mg. of nuclei.

4. Preparation of Liver and Kidney Nuclei To 50 gm. of liver tissue 500 ml of 2.3 M sucrose–0.001 M $CaCl_2$ or no $CaCl_2$ was added and the mixture blended for 10 minutes. The homogenate was strained through two layers of gauze. Nuclei were centrifuged at 21,000 R.P.M. for 80 minutes. The supernate was discarded. The pellets were collected and used for experiments. Calf kidney nuclei were prepared in the same way.

5. Preparation of Behrens' Thymus Nuclei The procedure for isolating thymus nuclei with non-aqueous media was that described by Allfrey *et al.* (41). In the present experiments, however, the frozen and dried tissue was suspended in petroleum ether and homogenized with a teflon plunger in the cold to prevent metal contamination. Whole frozen-dried tissue was also treated three times with the mixture of petroleum ether and carbon tetrachloride.

6. Incubation Procedure and Effects of Enzymes and Substances on Retention of Magnesium and Calcium in Ca- and Mg-Nuclei 1.0 ml. of nuclear suspension was mixed with 0.5 ml. of 0.1 M tris(hydroxymethyl)aminomethane buffer solution in 0.25 M sucrose and incubated aerobically at 37°C. for 30 minutes in a 7 ml. pyrex glass tube. Most of the experiments described were carried out at pH 6.65–6.74 excepting, of course, the experiments on the effects of varying the pH range. Every 10 minutes, the incubation mixture was gently mixed by hand. Instead of tris buffer–sucrose, 0.5 ml. of 0.1 M sodium phosphate buffer–sucrose was used in some experiments mentioned in the text. The final concentration of the buffer was 0.033 M and that of the $CaCl_2$ or $MgCl_2$ was 0.002 M, both in 0.25 M sucrose.

Metal contamination of the added enzymes and substances was tested for in each experiment.

Crystalline pancreatic deoxyribonuclease (Worthington Biochemical Corporation, once crystallized) and ribonuclease (Worthington Biochemical Corporation) were used. No contamination by calcium and magnesium was found in the enzyme preparations. The nucleic acids used were prepared by methods previously described (42, 43). Removal of contaminating metal from nucleic acid preparations was performed by the method described in the text. The denatured DNA was made by the method of Zubay (39). RNAase- and DNAase-split products of RNA and DNA digestion were prepared from the RNA and DNA from which metals had previously been removed with 10^{-1} M versene (pH 7.0). Histone I (arginine-rich) was prepared by methods previously described (44). The sodium salt of adenosine-5′-monophosphate was a preparation obtained from Schwartz Biochemicals Inc.

In testing the effects of DNAase, RNAase, and other substances, these substances were dissolved in buffer-sucrose at three times the final concentration desired. The final concentration of these substances is expressed as milligrams or micrograms per 1.5 ml. of the incubation mixture.

In studying the effect of DNAase on the release of calcium and magnesium from non-aqueous nuclei, each tube of the incubation mixture contained the following: 29.9 to 30.1 mg. nuclei, 2.0 ml. of 0.025 M sodium phosphate buffer–0.25 M sucrose, and 1.2, 1.0, 0.6, and 0.06 mg. of DNAase. In the control experiment, each tube contained the same amount of nuclei and 2.0 ml. of phosphate buffer–sucrose.

7. Effects of Acetate Buffer at Different pH's on Retention of Nucleotides, Magnesium, and Calcium in Ca- and Mg-Nuclei To 0.5 ml. of Ca-nuclei (Or Mg-nuclei) 4.5 ml. of 0.022 M acetate or succinate buffer solution in 0.25 M sucrose–0.003 M $CaCl_2$ (or $MgCl_2$) were added. The suspension was stirred and kept for 30 minutes at 1–2°C. After centrifugation at 5,000 R.P.M. for 5 minutes, the pH and ultraviolet absorption at 260 mμ of each supernate were measured by the procedure described by Osawa *et al.* (28).

Magnesium and calcium analyses were performed on the lipid-free dry sample (method of preparation described below in section 10) of the sediment obtained after centrifugation.

In testing the effects of adenosine-5′-diphosphate (Pabst Brewing Company) and -triphosphate (Sigma Chemical Company) upon the retention of magnesium in Ca-nuclei, these substances were dissolved in 1.0 ml. of a solution, 0.04 M acetate buffer (pH 6.60), 0.25 M sucrose, and 0.003 M $CaCl_2$. To 1.0 ml. of this nucleotide-containing solution 1.0 ml. of Ca-nuclei was added. The suspension was stirred and kept at 1–2°C for 30 minutes. The next step was to prepare lipid-free samples, as described below in section 10.

8. Effect of Removal of Sucrose on Retention of Magnesium and Calcium in Ca- and Mg-nuclei Substitution of glycerol or ethylene glycol for sucrose was carried out as follows: To 5.0 ml. of Ca-nuclei 100 ml. of 0.25 M glycerol- or ethylene glycol–0.003 M $CaCl_2$ were added and mixed. The nuclei were centrifuged at 3,000 R.P.M. for 7 minutes and the supernate discarded. The sediment was gently suspended in 100 ml. of the same medium. This procedure was repeated. Finally, the sediment was suspended in the same medium. The final volume was adjusted to 5.0 ml. and 1.0 ml. of the suspension was used to prepare a dry sample of the nuclei.

In testing the effects of tris buffer solution, Ca- or Mg-nuclei were suspended in 0.1 M tris buffer solution (pH 7.07–7.10)–0.003 M $CaCl_2$ or $MgCl_2$ just as in the case of glycerol or ethylene glycol.

All operations were carried out at 1–2°C.

9. Effect of Washing Nuclei with the Incubation Medium After incubation in the usual way, Ca-nuclei were centrifuged at 4,000 R.P.M. in the cold. The supernate was transferred to a 20 ml. calibrated test tube. To the sedimented nuclei was added 1 ml. of the usual incubation medium (2 volumes of 0.25 M sucrose–0.003 M $CaCl_2$ and 1 volume of 0.1 M tris buffer–0.25 M sucrose). The nuclei were gently stirred and then 4 ml. more of the medium were added and the suspension gently stirred again. The suspension was centrifuged at 5,000 R.P.M. for 10 minutes. This supernate was added to the previous supernate. This procedure was repeated two or three times. During the second or third washing, there was a slight gel formation. All operations were carried out at 1–2°C.

Measurement of the mononucleotides released during washing was carried out as described by Osawa *et al.* (28).

10. Preparation of Dry Lipid-Free Samples After incubation, a nuclear suspension was centrifuged in a tared tube at 4,000 R.P.M. for 7 minutes in the cold. The super-

nate was decanted and discarded. The sediment was suspended in 7 ml. of 95 per cent alcohol and kept at 60°C. for 5 minutes with occasional stirring. After centrifugation, the sediment was resuspended in 7 ml. of 88 per cent alcohol (to remove salts and sucrose) and again kept at 60°C. This procedure was repeated twice and then the sediment was again suspended in hot 95 per cent alcohol for 5 minutes. Finally the sediment was washed twice with ether and then kept overnight in a vacuum desiccator.

Lipid-free samples of tissues were prepared in the same way.

11. Ashing To about 30 mg. of the lipid-free dry sample in a 7 ml. pyrex tube 2 ml. of perchloric acid (1 volume)–nitric acid (2 volume) mixture and a small piece of teflon were added. The tubes were kept for 1 to 2 hours just above a melted salt bath the temperature of which was 140°C. After the samples had completely dissolved, the tubes were gradually lowered into the bath. Then, heating was continued for 6 to 7 hours at 210 to 230°C. When all fluids had evaporated, to the ash remaining at the bottom of the tube 1 ml. of the acid mixture was again added and the samples were re-ashed in the same way. Finally heating was continued for 3 hours more at 300°C., the ash now being altogether white.

The ash was dissolved in 0.2 ml. of 2 N HCl (heating, if necessary, to dissolve) and diluted to 2.0 ml. with double distilled water.

12. Calcium Determination Analysis of calcium was performed by the procedure of Rich (45) on the dissolved ash. An amount of the sample which contained between 20 and 100 μg. calcium was used. To the sample double distilled water was added to bring the volume to 2.5 to 3.0 ml. Then, 2 drops of methyl red indicator were added and the solution was brought to pH 4.5 with dilute ammonium hydroxide and acetic acid. To this sample 2 ml. of 3 per cent ammonium oxalate were added, mixed, and allowed to stand overnight. 2 drops of 2 per cent triton solution were added to obtain satisfactory sedimentation on centrifugation and the precipitate of oxalate was centrifuged. The supernate was discarded. 0.1 ml. of 2 N HCl was added to dissolve the precipitate and then 0.9 ml. of double distilled water was added. 2.5 ml. of 2 N NaOH and 0.2 ml. of 0.025 per cent plasmo corinth B (Sumitomo Chemical Company) solution (46) were added within 5 minutes of the time of titration. Titration was carried out with successive additions of 0.02 to 0.10 ml. of a versene solution (180 mg. disodium ethylenediaminetetraacetate per 1000 ml. of double distilled water) using a Coleman spectrophotometer set at 610 mμ. The end point was measured by plotting increments of optical density after additions of the versene solution. The blank and standard solutions were also titrated in the same way.

13. Magnesium Determination Analysis of magnesium was performed by the procedure of Orange and Rhein (47). An amount of the dissolved ash containing 2.5 to 7.5 μg. of magnesium was transferred to a 20 ml. test tube. The sample was diluted to 1.0 ml. with double distilled water and the following reagents were added: (*a*) 1.0 ml. of 0.05 per cent cupferron solution. The tube was shaken vigorously. Then the cupferron-iron complex was extracted with ether three times. Finally the remaining ether was evaporated completely. (*b*) 1.0 ml. of freshly prepared 0.2 per cent poly-

vinyl alcohol. (*c*) 1.0 ml. of 0.0075 per cent Titan yellow (Amend Drug and Chemical Company) solution. (*d*) 1.0 ml. of 15 per cent NaOH. Immediately absorptions were read in the Beckman spectrophotometer at 560 mμ. Both blank and standard were prepared in the same way and at the same time, substituting either 1.0 ml. of double distilled water for the blank or 1 ml. of an appropriate standard. The graph for known values was drawn in each determination and then the unknowns read from it. It was observed that Beer's law holds for concentrations up to 10 μg. of magnesium.

When Ca-nuclei were used for analysis of magnesium, the possibility of interference in the color development of the magnesium-dye complex by calcium must be considered (48, 49) since the nuclei contain a large amount of calcium. To test the validity of the magnesium method in the presence of calcium, 2.50, 5.00, and 7.50 μg. of magnesium were added to a series of solutions containing 0 to 600 μg. calcium. It was observed that the presence of calcium in the solution to be analyzed affects the color development, if the calcium-magnesium ratio is greater than 40 to 1. The presence of excess calcium decreased the optical density, so that the amount of magnesium found was lower than the expected value. In the determination of 2.50 to 7.50 μg. of magnesium, 100 μg. of calcium per ml. of sample can be present without affecting the measurement by more than 4 per cent. In the present experiments the calcium content of a sample analyzed for magnesium has been in general less than 60 μg.

14. Nucleic Acid Analyses In all cases, nucleic acids analyses were performed on the lipid-free dry samples. Dry nuclei were washed twice with cold 5 per cent trichloracetic acid or cold 2 per cent perchloric acid to removal small nucleotides. When cold perchloric acid was used, nuclei were twice washed with 95 per cent alcohol before being extracted with hot trichloracetic acid. Nucleic acids were extracted from nuclei with 5 per cent trichloracetic acid at 90°C. The amount of DNA was determined by the diphenylamine reaction (51). RNA determination was performed by the procedure of Webb (50).

This work was supported in part by The United States Public Health Service grant, RG-4919.
Received for publication, July 25, 1960.

REFERENCES

1. Data for Biochemical Research, (R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones, editors), Oxford, The Clarendon Press, 1959.
2. KORNBERG, A., *Harvey Lectures*, 1959, **53**, 83.
3. GRUNBERG-MANAGO, M., ORTIZ, P. J., and OCHOA, S., *Biochim. et Biophysica Acta*, 1956, **20**, 269.
4. HOAGLAND, M. B., KELLER, E. B., and ZAMECNIK, P. C., *J. Biol. Chem.*, 1956, **218**, 345.
5. HUISKAMP, W., *Z. physiol. Chem.*, 1901, **32**, 145.
6. CARTER, R. O., and HALL, J. L., *J. Am. Chem. Soc.*, 1940, **62**, 1194.
7. ALLFREY, V. G., MIRSKY, A. E., and OSAWA, S., *J. Gen. Physiol.*, 1957, **40**, 451.

8. MAZIA, D., *Proc. Nat. Acad. Sc.*, 1954, **40,** 521.
9. LEVINE, R. P., *Proc. Nat. Acad. Sc.*, 1955, **41,** 727.
10. EVERSOLE, R. A., and TATUM, E. L., *Proc. Nat. Acad. Sc.*, 1956, **42,** 68.
11. CHAO, F. C., *Arch. Biochem. and Biophysic.* 1957, **70,** 426.
12. Ts'O, P. O., BONNER, J., and VINOGRAD, J., *Biochim. et Biophysica Acta*, 1958, **30,** 570.
13. ALLFREY, V. G., *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, Inc., 1959, **1,** 193.
14. ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1957, **43,** 821.
15. ALLFREY, V. G., AND MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1957, **43,** 589.
16. SCHNEIDER, R. M., and PETERMAN, M. L., *Cancer Research*, 1950, **10,** 751.
17. WILLIAMSON, M. D., and GULICK, A., *J. Cell. and Comp. Physiol.*, 1944, **23,** 77.
18. CHAUVEAU, J., MOULÉ, Y., and ROUILLER, C., *Exp. Cell Research*, 1956, **11,** 317.
19. ALLFREY, V. G., and MIRSKY, A. E., unpublished observation.
20. Handbook of Biological Data, (W. S. Spector, editor), Philadelphia, W. B. Saunders Co., 1956.
21. SCHNEIDER, R. M., *Exp. Cell Research*, 1955, **8,** 24.
22. MAZIA, D., *in* Trends in Physiology and Biochemistry, (E. S. G. Barrón, editor), New York, Academic Press, Inc., 1952.
23. WEISSMAN, N., and FISHER, J., *J. Biol. Chem.*, 1949, **178,** 1007.
24. KUNITZ, M., *J. Gen. Physiol.*, 1950, **33,** 363.
25. SINSHEIMER, R. L., *J. Biol. Chem.*, 1954, **208,** 445.
26. SHACK, J., and BYNUM, B. S., *Nature*, 1959, **184,** 635.
27. FELSENFELD, G., and HUANG, S., *Biochim. et Biophysica Acta*, 1959, **34,** 234.
28. OSAWA, S., ALLFREY, V. G., and MIRSKY, A. E., *J. Gen. Physiol.*, 1957, **40,** 491.
29. EPP, A., RAMASARMA, T., and WETTER, L. R., *J. Am. Chem. Soc.*, 1958, **80,** 724.
30. WACKER, W. E., and VALLEE, B. L., *J. Biol. Chem.*, 1959, **234,** 3257.
31. STAEHELIN, M., *Experientia*, 1959, **15,** 413.
32. POLICARD, A., *Compt. rend. Acad. sc.*, 1923, **176,** 1012.
33. POLICARD, A., and PILLET, D., *Compt. rend. Soc. biol.*, 1928, **98,** 1350.
34. SCOTT, G. H., *Am. J. Anat.*, 1933, **53,** 243.
35. SCOTT, G. H., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32,** 1428.
36. THIERS, R. E., and VALLEE, B. L., *J. Biol. Chem.*, 1957, **226,** 911.
37. STEFFENSEN, D., and BERGERON, J. A., *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 339.
38. ZUBAY, G., and DOTY, P., *Biochim. et Biophysica Acta*, 1958, **29,** 47.
39. ZUBAY, G., *Biochim. et Biophysica Acta*, 1959, **32,** 233.
40. ALLFREY, V. G., and MIRSKY, A. E., *Proc. Nat. Acad. Sc.*, 1958, **44,** 981.
41. ALLFREY, V. G., STERN, H., MIRSKY, A. E., and SAETREN, H., *J. Gen. Physiol.*, 1952, **35,** 529.
42. MIRSKY, A. E., and POLLISTER, A. W., *Proc. Nat. Acad. Sc.*, 1942, **28,** 344.
43. DALY, M. M., ALLFREY, V. G., and MIRSKY, A. E., *J. Gen. Physiol.*, 1950, **33,** 497.
44. DALY, M. M., and MIRSKY, A. E., *J. Gen. Physiol.*, 1955, **38,** 405.
45. RICH, G., personal communication.

46. YANAGIZAWA, F., *in* Standard Methods of Biochemistry, (F. Egami, editor), Tokyo, Kôbundo, 1953.
47. ORANGE, M., and RHEIN, H. C., *J. Biol. Chem.*, 1951, **189**, 379.
48. SNELL, F. S., and SNELL, C. T., Colorimetric Methods of Analysis, New York, D. Van Nostrand Co., Inc., 1959, **2A**.
49. SANDELL, E. B., Colorimetric Determination of Tracers of Metals, New York, Interscience Publishers Inc., 1944.
50. WEBB, J. M., *J. Biol. Chem.*, 1956, **221**, 635.
51. DISCHE, Z., *Mikrochemie* 1930, **8**, 4.