# MONONUCLEOTIDES OF THE CELL NUCLEUS

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It has been shown in the preceding paper (1) that protein synthesis occurs within the cell nucleus. The question that then naturally arises is how the nucleus meets the energy requirement for the synthetic process. Since it has been generally accepted that in the cytoplasm energy-rich compounds such as ATP and related nucleotides participate in numerous synthetic processes, one might suppose that such compounds are involved in protein synthesis within the nucleus. It has, in fact, also been shown in the preceding paper (1) that protein synthesis in the nucleus is an oxidative process and is blocked by certain "uncoupling" reagents which are known to inhibit the synthesis of ATP in mitochondria. We have therefore investigated the nucleotide system of the nucleus and this will be described in the present paper.

Our first step has been to show that quantitatively and qualitatively much the same complement of acid-soluble mononucleotides occurs in the nucleus as in the cytoplasm. Then it has been found that within the nucleus these nucleotides can be phosphorylated to the energy-rich triphosphate form. As to the nature of the phosphorylation, there exist certain resemblances as well as differences between the nucleus and mitochondria. Another aspect of the problem that has been investigated concerns the way in which nucleotides are held in the nucleus. Finally, attempts have also been made to elucidate the role of the nuclear mononucleotides in the synthesis of protein as it occurs in the nucleus.

The work which will be mentioned below has been done on calf thymus nuclei isolated in sucrose media, unless otherwise specified. A preliminary account of this work has been published (2).

*Presence of Nucleotides in the Nucleus.*—When one investigates water-soluble substances such as mononucleotides in isolated nuclei, it is important to use preparations isolated in such a way that loss or adsorption of these substances is prevented during the course of nuclear isolation. Nuclei isolated in non-aqueous media (so called Behrens' type nuclei (3)) meet these requirements. Analyses of nucleotides were therefore first performed on isolated "non-aqueous" nuclei of calf thymus, and compared with the whole tissue treated

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with the same media to which the nuclei were exposed during their isolation. Cold 2 per cent perchloric acid extracts of non-aqueous nuclei and tissue were fractionated by dowex 1 ion exchange chromatography as described by Cohn and modified by Hurlbert *et al.* (4). A typical chromatogram is illustrated in

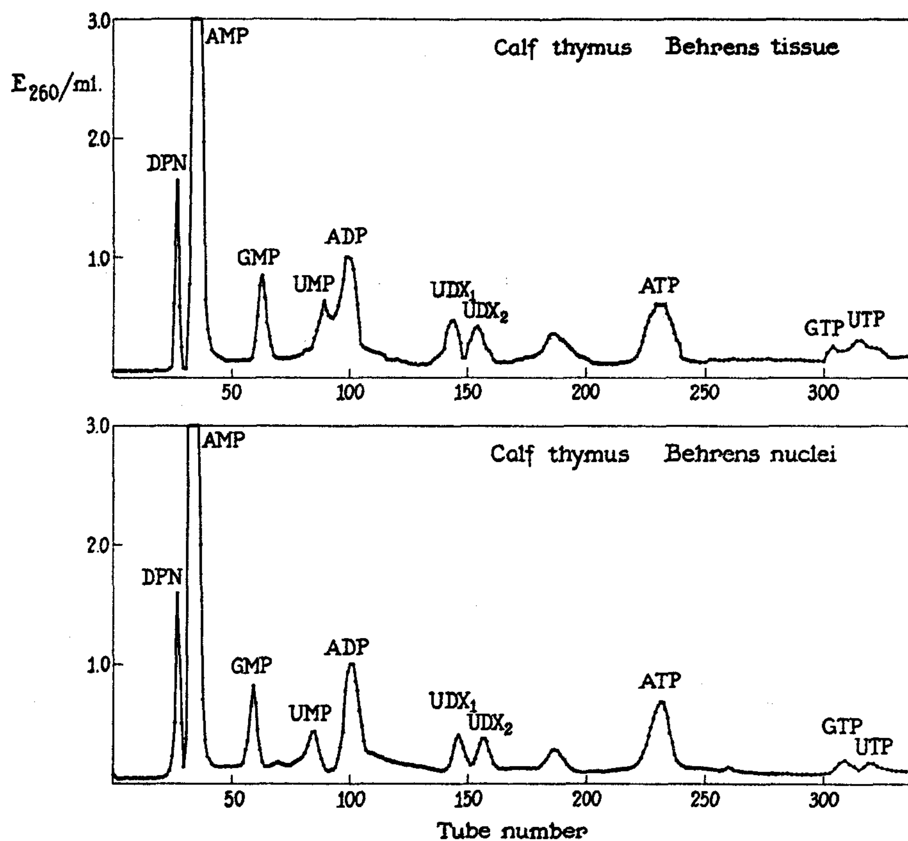


FIG. 1. Acid-soluble nucleotides of calf thymus non-aqueous nuclei and the corresponding whole tissue (see also the top column of Table I). Note the predominance of monophosphates over di- and triphosphates.

Fig. 1. It is seen that calf thymus nuclei contain quantitatively and qualitatively nearly the same complement of nucleotides as the whole tissue. Mononucleotides of adenine, guanine, cytosine, and uracil, together with several uridine diphosphate derivatives and diphosphopyridine nucleotide were identified; one of the striking features here is the predominating amount of adenine nucleotides. Only a trace of cytidine nucleotides was found. In a 2 per cent perchloric acid extract more than 80 per cent of ultraviolet-absorbing material

at 260 mμ is found to consist of nucleotides either by adding up all nucleotide peaks on the chromatogram, or by measuring at 260 mμ the quantity of light-absorbing material which did not combine with dowex 1 resin. The quantity of nucleotides in non-aqueous thymus nuclei is about 1.7 per cent of the total nuclear mass. Practically the same content of nucleotides in the nucleus as in

TABLE I

*Acid-Soluble Adenine Nucleotides in Tissues and Nuclei*

| Material analyzed | $E_{260}$ per gm. lipide-free dry matter present as | | | | |
|---|---|---|---|---|---|
| | Total $E_{260}$ | AMP | ADP | ATP | Total adenine nucleotides |
| Calf thymus tissue* (Behrens' preparation) | 500 | 102 | 56 | 43 | 201 |
| Calf thymus Behrens' nuclei* | 500 | 93 | 58 | 44 | 195 |
| Calf thymus Behrens' nuclei | 420 | 80 | 19 | 14 | 113 |
| Calf thymus tissue | 580 | 119 | 29 | 15 | 163 |
| Calf thymus tissue | 580 | 118 | 33 | 20 | 171 |
| Calf thymus sucrose nuclei | 306 | 28 | 23 | 46 | 97 |
| Calf thymus sucrose nuclei | 390 | 34 | 22 | 58 | 104 |
| Calf thymus sucrose nuclei | 460 | 39 | 29 | 89 | 157 |
| Calf liver tissue (Behrens' preparation) | 382 | — | — | — | — |
| Calf liver Behrens' nuclei | 350 | — | — | — | — |
| Chicken erythrocytes (Behrens' preparation) | 92.0 | 3.2 | 9.3 | 15.8 | 28.3 |
| Chicken erythrocyte (Behrens' nuclei) | 95.4 | 6.1 | 14.0 | 14.1 | 34.2 |
| Brown trout sperm | 170 | — | — | — | — |
| Rainbow trout sperm | 171 | — | — | — | — |

* Derived from the same thymus gland.

the cytoplasm was also found in the case of calf liver and of chicken erythrocytes, indicating the general occurrence of the nucleotide system in cell nuclei. The over-all concentration of nucleotides in the nucleus is, however, different from one type of tissue to another. Nuclei from metabolically active tissues such as calf thymus, or calf liver have a higher content of nucleotides than is found in nuclei of such metabolically inert cells as chicken erythrocytes or trout spermatozoa (Table I).

When analyses of nucleotides are performed on calf thymus nuclei isolated

in 0.25 M sucrose–0.002 to 0.003 M $CaCl_2$, it is found that they usually retain 60 to 80 per cent of their original acid-soluble nucleotide content. However, the ability to retain the nucleotides in sucrose nuclei from different calf thymus tissues at different times of the year varies considerably. Such nucleotides as are retained after isolation of nuclei remain in the nuclei even after repeated washings with cold sucrose–$CaCl_2$ solution (Table II). This is rather surprising

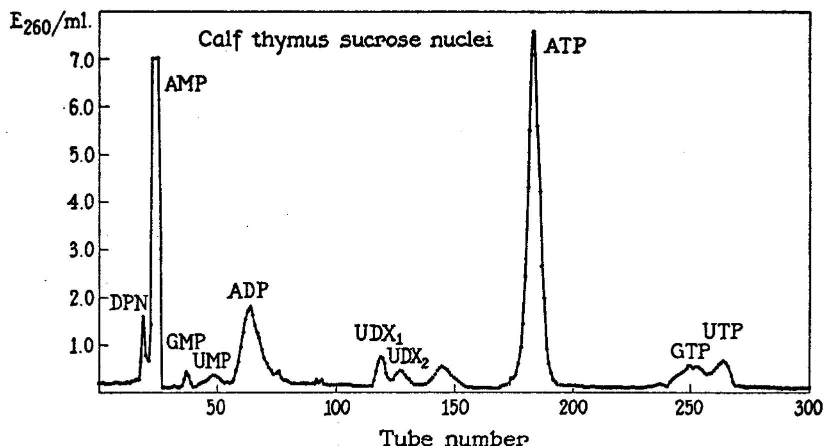


FIG. 2. Acid-soluble nucleotides of calf thymus sucrose nuclei. Note the predominance of triphosphates over di- and monophosphates.

TABLE II

*Effect of Sucrose Washing on Nuclear Acid Soluble Nucleotides in Calf Thymus Sucrose Nuclei*

| | $E_{260}$ per gm. lipide-free dry matter | | |
|---|---|---|---|
| | Experiment 1 | Experiment 2 | Experiment 3 |
| Original sucrose nuclear preparation | 264 | 198 | 100 |
| After 1st washing | 234 | 177 | 90 |
| After 2nd washing | 232 | — | — |

because of the diffusible nature of nucleotides in aqueous media, and because the nuclear membrane of nuclei isolated in a sucrose solution is not a barrier to the passage of even much larger molecules. The way in which nucleotides are retained in the nucleus will be discussed later in this paper. Chromatographic analyses (Fig. 2, Table I) revealed that sucrose nuclei have a pattern of nucleotide distribution similar to that found in non-aqueous nuclei, with an important exception which will be dealt with in the next section.

Even before the analyses of nucleotides on isolated nuclei that have just been described were made, there were observations suggesting the presence of

these substances in nuclei: Davies (5), and Walker and Yates (6) in the course of their microspectrophotometric observations on nuclei in tissue culture, reported that there are substances which absorb ultraviolet light and are soluble in acid-alcohol fixatives. The English authors supposed that their "lower nucleotides" are precursors of DNA. However, their procedure does not permit identification of each nucleotide. Naora and Takeda (7) recognized the occurrence of labile phosphate compounds, which they assumed to be ATP, in the rat liver nuclei isolated by the Behrens' procedure. Although possible contamination of glucose-1-phosphate which is also acid-labile (8) and is known to occur in the nuclei should be considered, at least a part of their "labile phosphate" must be ATP. Edmonds and LePage (9) reported the presence of various nucleotides in the sucrose nuclei obtained from Flexner-Jobling tumor cells. Kay and Davidson (10) noted the occurrence of acid-soluble nucleotides in non-aqueous nuclei from various tissues. Now their chemical nature has been established and it is likely that nuclear mononucleotides, as cytoplasmic ones, play a part in numerous processes, including the synthesis of protein that occurs in the nucleus.

*Phosphorylation of Nucleotides in the Nucleus.*—The predominance of nucleotide monophosphates in thymus tissue and in thymus nuclei prepared by the non-aqueous procedure should not be taken to indicate the actual condition *in vivo*, or an artifact due to the action of non-aqueous media. We have repeatedly observed that socalled "fresh" calf thymus tissue has a similar predominance of monophosphates over di- and triphosphates (Table I). It seemed probable to us that the predominance of these low energy forms is simply a reflection of anoxia resulting from the death of the animal. Evidence to support this conclusion is derived from a series of nucleotide analyses of rat thymus tissues which can be excised from the animal after death with a speed that cannot be achieved in the case of a slaughter-house animal such as the calf. Some of the rat tissues were frozen in liquid nitrogen immediately upon excision; others were incubated at 30°C. for 5, 10, and 20 minutes. As can be seen in Fig. 3, in the immediately frozen tissue ATP predominates over ADP and AMP, and during the course of incubation the amount of ATP sharply drops with a simultaneous increase of AMP, ADP content remaining relatively unchanged. The same sort of changes were observed in the uridine and guanine nucleotides. A comparison of nucleotide distribution in non-aqueous nuclei and sucrose nuclei reveals an interesting difference between them: the predominance of low energy forms in non-aqueous nuclei and of high energy forms in sucrose nuclei. This is taken to indicate that during the preparation of sucrose nuclei in the cold, the monophosphate characteristic of the excised tissue is largely converted back to the energy-rich triphosphate form which predominates in the living tissue. The post-mortem predominance of nucleotide monophosphates in thymus tissue seems

therefore to afford a unique opportunity to study the mechanism of their phosphorylation in the nucleus.

The formation of high energy phosphate bonds is presumably the result of extensive aeration of the homogenates and nuclear suspensions during the isolation procedure. To determine whether this phosphorylation involves participation of the cytoplasmic fraction in the homogenate, or whether it is an intranuclear phenomenon, two types of experiments were carried out.

1. Nuclei were isolated as quickly as possible (at 2°C. in 25 to 28 minutes). At this time most nucleotides are still present in the monophosphate form.

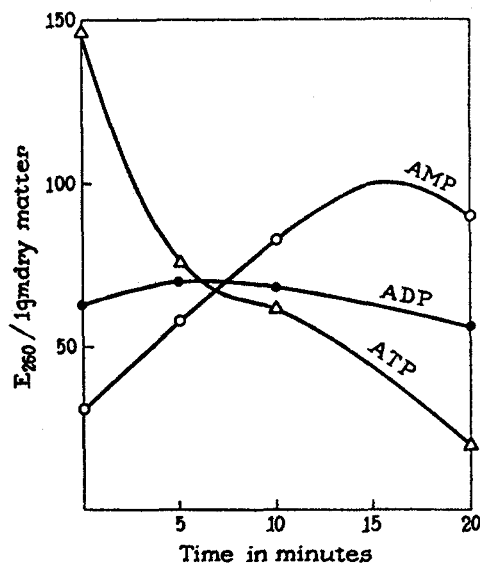


FIG. 3. Postmortem changes of adenine nucleotides in rat thymus tissue. Excised thymus tissue was incubated at 30°C. for 5, 10, 15, and 20 minutes.

Such a nuclear preparation was then gently stirred for an additional 50 to 60 minutes in the cold. At the end of this time a large part of the monophosphates had been converted into triphosphates (Table III, Fig. 4).

2. A thymus tissue homogenate in sucrose–$CaCl_2$ solution was stirred gently for 95 minutes at 4°C. This homogenate was then fractionated into nuclear and cytoplasmic components. As indicated in Table IV, the original homogenate at the beginning of the experiment contained a large amount of AMP but almost no ATP. It is obvious that during the incubation of the homogenate in the cold under the prevailing experimental conditions, phosphorylation of AMP took place in the nuclear but not in the cytoplasmic fraction, and the same may be said of the guanine and uridine nucleotides. The decrease of AMP content in the cytoplasmic fraction was probably the result of phos-

phatase action. This phosphatase does not seem to act on the nuclear nucleotides (Table IV).

The two experiments that have just been described show that phosphorylation of nucleotides occurs within the nucleus. The next experiments were arranged to enable us to compare nuclear phosphorylation with mitochondrial oxidative phosphorylation, which has been extensively investigated. An important characteristic of phosphorylation in mitochondria is that considerable quantities of nucleotides can be added to a mitochondrial preparation and that the nucleotides so added become phosphorylated. A distinctive feature of nuclear phosphorylation is that only intranuclear monophosphates seem to be phosphorylated, for added AMP is not acted upon. In an experiment to show whether nuclei act upon added nucleotides, thymus sucrose nuclei were isolated rapidly so that most nucleotides were still in the form of mononucleotides. At this point, to the concentrated nuclear suspension, about twice as much AMP as total intranuclear nucleotides was added with and also without the appropriate amount of inorganic phosphate. The suspension was then stirred for an additional 120 minutes in the cold. At the end of the experiment, exactly the same amount of AMP that had been added

TABLE III

*Phosphorylation in Isolated Calf Thymus Sucrose Nuclei*

| Material analyzed | Total $E_{260}$ in 1 gm. dry matter | Per cent of total $E_{260}$ present as | | | |
|---|---|---|---|---|---|
| | | AMP | ADP | ATP | Total adenine nucleotides |
| Sucrose nuclei after rapid isolation (25 min. at 2°C.) | 308 | 19.0 | 10.5 | 7.6 | 37.1 |
| Above nuclei stirred at 2°C. for 50 min. after isolation | 308 | 3.5 | 5.7 | 24.5 | 33.7 |
| Sucrose nuclei after rapid isolation (28° min. at 2°C.) | 295 | 14.2 | 8.5 | 7.6 | 30.3 |
| Above nuclei stirred gently at 2°C. for 60 min. after isolation | 300 | 4.6 | 5.6 | 20.4 | 30.4 |
| Calf thymus tissue | — | 18.8 | 5.8 | 3.7 | 28.3 |
| Sucrose nuclei prepared rapidly from above tissue (28 min. at 2°C.) | 240 | 16.0 | 7.9 | 6.7 | 30.6 |
| Above nuclei stirred gently at 2°C. for 60 min. after isolation | 233 | 6.3 | 6.4 | 16.6 | 29.3 |

to the suspension was recovered as AMP, whereas the amount of ADP, and the amount of synthesized ATP were just the same in the material to which AMP had been added and in the control to which no AMP had been added. It should be noted that the phosphate required for phosphorylation is avail-

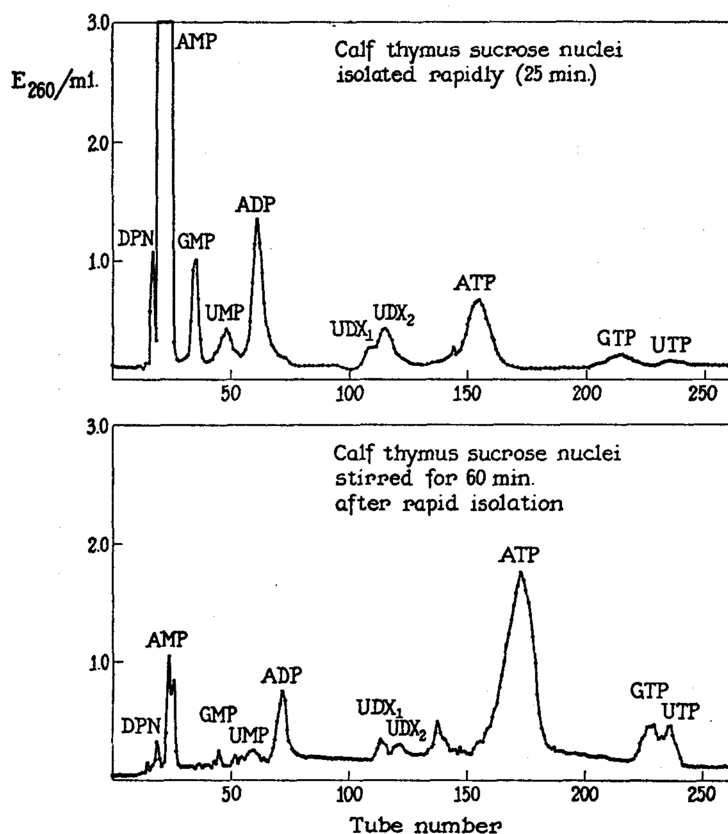


FIG. 4. Phosphorylation in isolated calf thymus sucrose nuclei. Acid-soluble nucleotides of sucrose nuclei isolated rapidly (in 25 minutes) at 2°C. (above), and of the same nuclei stirred gently in the cold for an additional 50 minutes (below). Note the increase in amount of triphosphates after 50 minutes.

able inside the nucleus; in no experiments on nuclear phosphorylation has the addition of phosphate been required. We have made some preliminary observations in this connection; sucrose nuclei when isolated rapidly contain less inorganic phosphate than is required for the phosphorylation of nuclear mononucleotides, and during the course of phosphorylation inorganic phosphate actually increases in amount. It is therefore probable that the phosphate available for the nuclear phosphorylation is derived from some organic phosphates in the nucleus.

When the effects of metabolic inhibitors are tested, one can see certain resemblances as well as differences between nuclear and mitochondrial phos-

TABLE IV

*Synthesis of ATP in the Sucrose Homogenates of Calf Thymus Tissue with Separation into Nuclear and Cytoplasmic Fractions*

| Material analyzed | Per cent of total $E_{260}$ present as | | | | | |
|---|---|---|---|---|---|---|
| | Unadsorbed to dowex 1 | AMP | ADP | ATP | Total adenine nucleotides | Unadsorbed + total adenine nucleotides |
| Homogenate | 18.0 | 14.5 | 3.1 | 1.5 | 19.1 | 37.1 |
| Cytoplasm | 28.0 | 7.7 | 2.3 | 1.0 | 11.0 | 39.0 |
| Nuclei | 18.0 | 2.5 | 3.2 | 14.4 | 20.1 | 38.1 |

TABLE V

*Effects of Metabolic Inhibitors on Phosphorylation of Nucleotides in Isolated Calf Thymus Sucrose Nuclei*

| Inhibitor | Concentration | Change in phosphorylation due to added inhibitor | Inhibitory effect on | | References for mitochondrial data |
|---|---|---|---|---|---|
| | | | Nuclear phosphorylation | Mitochondrial phosphorylation | |
| | | *per cent* | | | |
| Sodium cyanide | $1 \times 10^{-3}$ M | −100 | + | + | — |
| Carbon monoxide | 100 per cent | −100 | + | + | — |
| 2,4-Dinitrophenol | $2 \times 10^{-4}$ M | −100 | + | + | Loomis and Lipmann (1948) |
| Sodium azide | $1 \times 10^{-3}$ M | −100 | + | + | Loomis and Lipmann (1949) |
| Antimycin A | 1 μg./ml. | −61 | + | + | Potter and Reif (1952) |
| Dicumarol | $1 \times 10^{-4}$ M | 0 | − | + | Martius and Nitz-Litzow (1953) |
| Janus green B | $2 \times 10^{-5}$ M | +6 | − | + | Dianzani and Scuro (1956) |
| Methylene blue | $2 \times 10^{-5}$ M | +30 | − | + | Lehninger (1949) |
| | $2.5 \times 10^{-4}$ M | +21 | | | |
| Calcium ions | $2 \times 10^{-3}$ M | 0 | − | + | Lehninger (1949) |
| | $3 \times 10^{-3}$ M | 0 | | | |
| | $4 \times 10^{-3}$ M | 0 | | | |

phorylation (Table V). Nuclear, like mitochondrial phosphorylation, is completely inhibited by some common inhibitors such as sodium cyanide, sodium azide (11), and dinitrophenol (12). Considering the mode of action of these inhibitors it is probable that the phosphorylation concerned is oxidative, and involves electron transport by means of cytochrome whereby energy-rich phos-

phate bonds are synthesized. Antimycin A (13) blocked this process by 60 per cent. Calcium ions are necessary in our system in order to maintain the nuclear structure. Varying the amount of calcium concentrations, from $2 \times 10^{-3}$ M to $4 \times 10_0^{-3}$ M, did not influence the rate of phosphorylation. That no phosphorylation takes place in the cytoplasmic fraction under our experimental conditions has been mentioned already. Phosphorylation in the cytoplasmic fraction is probably prevented by the presence of calcium ions, a known potent inhibitor of mitochondrial oxidative phosphorylation (14). Certain reagents which block phosphorylation in mitochondria, namely dicumarol (15), Janus green B (16, 17), and methylene blue (14, 15), have no effect (if any, slightly accelerative) on nuclear phosphorylation. These observations may be taken as additional evidence against the participation of mitochondria in the phosphorylation occurring in preparations of isolated nuclei. In all inhibitor experiments, the adenine, uridine, and guanine nucleotides behaved in a parallel fashion.

Nuclei do not seem to contain the equipment for ordinary terminal oxidation as found in mitochondria, such as cytochrome *c* oxidase, cytochrome *c* and the flavoproteins including cytochrome *c* reductases (18, 19). In the case of calf thymus it has been reported that cytochrome *c* oxidase is sharply localized in the mitochondrial fraction; no detectable amount of this enzyme was found in the nuclei (19). The evidence that cytochrome *c* oxidase is absent from the nuclei is, however, perhaps not as decisive as would appear. The fact that the cytochrome *c* added to nuclei is not oxidized should be considered in relation to the fact that added AMP is also not acted upon. And yet in the latter case we know that nuclei actually phosphorylate the AMP already within them. It is, accordingly, worth considering that nuclei may contain cytochrome *c*, possibly in low concentration, which they are able to oxidize. Experiments on DPN cytochrome *c* reductase are unfortunately not decisive (19). If sucrose nuclei do not in fact contain any cytochrome *c* oxidase, it may be supposed that nuclear phosphorylation proceeds through a respiratory chain which differs from that present in mitochondria. One such difference might be the occurrence of different cytochromes, and in this connection it has already been shown that the cytochromes of microsomes differ from those of mitochondria (20, 21). Although inhibitor experiments indicate a difference between nuclear and mitochondrial phosphorylation, they do not yet throw any light on the details of the nuclear system.

Considering the fact that cyanide, azide, and dinitrophenol block nuclear phosphorylation the involvement of glycolysis in this process is unlikely.

Although most inhibitors which block nuclear phosphorylation also stop protein synthesis in the nucleus, at least one reagent, namely dicumarol, which does not affect phosphorylation does inhibit protein synthesis (1).

This result might indicate that dicumarol blocks the energy transfer from the nucleotide system to the synthesis of protein.

*Retention of Nucleotides in the Nucleus.*—The ability of sucrose nuclei to retain nucleotides is really quite surprising. It has been mentioned already that the bulk of the nucleotides is retained in the sucrose nuclei after their isolation. The nucleotides of isolated nuclei are well retained for at least 24 hours in the cold, or after repeated washings of nuclei with cold sucrose–$CaCl_2$ solution. Treatment of sucrose nuclei with 0.5 M sucrose, or with 0.25 M sucrose–0.003 M $CaCl_2$ containing 0.14 M NaCl has almost no effect on nucleotide retention.

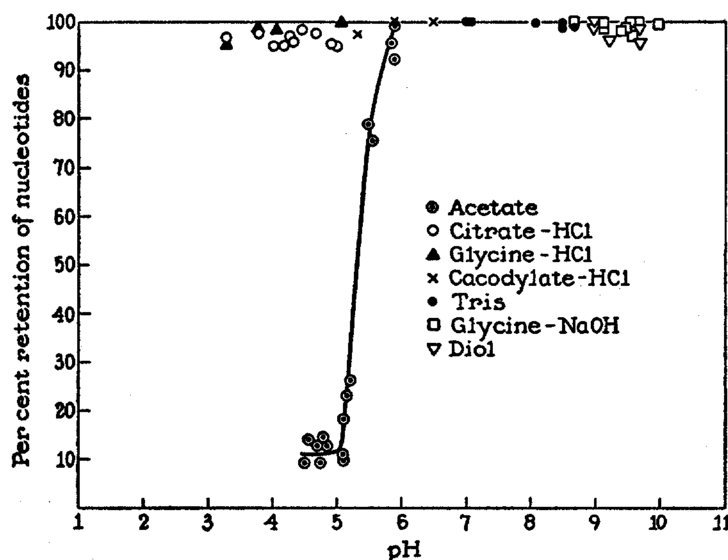


FIG. 5.

Nucleotide retention in the nuclei is, however, dependent on the presence of sucrose, for as soon as sucrose is replaced by a buffer solution in water at any pH, all nucleotides are completely lost from the nuclei. Citric acid nuclei, sucrose nuclei treated with 0.01 M citric acid without sucrose, or sucrose nuclei treated with saline in the absence of sucrose do not contain the nucleotides. One of the interesting features about nucleotide retention may be its irreversibility. Once nuclei lose their nucleotides by any means we have tried, nucleotides added to the medium cannot be taken up.

As long as there is sucrose in the medium, nuclei can be subjected to dilute buffer solutions over a wide pH range without loss of nucleotides (Fig. 5). In such experiments, to 1 volume of nuclear suspension, 9 volumes of 0.022 M buffer in 0.25 M sucrose–0.003 M $CaCl_2$ were added and kept in the cold for

30 minutes. After centrifuging, the pH and extinction at 260 m$\mu$ of the supernatant were measured. At least 95 per cent of nucleotides were retained over the whole pH range tested (pH 3.25 to 10.0). Buffers used included glycine-HCl, citrate-HCl, cacodylic acid–NaOH, "tris", "diol," and glycine-NaOH.

When sucrose nuclei were treated with a series of acetate buffers in sucrose solution, a surprising observation was made. Dilute acetate buffer (0.02 M) removed 85 to 90 per cent of the nucleotides from the sucrose nuclei below pH 5.1. In order to learn how specific acetate is in releasing nucleotides from nuclei in a sucrose medium, some related substances were tested. The results are included in Table VI. It is clear that certain simple fatty acids such as formic, propionic, and monochloroacetic have a similar ability to release nucleotides. However, ethyl alcohol, acetaldehyde, citrate, glycine-HCl, succinate, and acetone have no effect on the removal of nucleotides from the sucrose nuclei.

TABLE VI

*Effect of Some Substances on the Retention of Nucleotides in Calf Thymus Sucrose Nuclei*

| Substances 0.02 M in sucrose | pH of media | Per cent loss |
|---|---|---|
| Formate | 4.65 | 98 |
| Acetate | 4.45 | 90 |
| Acetate | 5.90 | 1.9 |
| Propionate | 4.32 | 95 |
| Monochloroacetate | 4.35 | 99 |
| Ethyl alcohol | 4.25 | 2.8 |
| Acetaldehyde | 4.40 | 4.1 |
| Citrate-HCl | 4.45 | 2.5 |
| Glycine-HCl | 4.10 | 0 |
| Succinate | 4.35 | 0 |
| Acetone | 4.35 | 0 |
| Acetone | 7.45 | 0 |

As to the effect of acetate, it is obvious from Fig. 5 that there is a sharp boundary of effective pH of acetate between pH 5.1 and pH 5.9. In this pH range, the quantity of nucleotides released by acetate gradually increases as the pH drops. At pH 5.9 there is no loss of nucleotides by acetate; at pH 5.1 the maximum loss occurs.

A time course study on the effect of dilute acetate (0.005 M) at pH 4.5 (see Fig. 4) revealed that 90 per cent of the nucleotides were released within less than 2 minutes without further increase over 30 minutes; at pH 5.9 there is no sign of nucleotide release by acetate even after 30 minutes. These facts clearly indicate that release of nucleotides from the nuclei by acetate is pH-dependent. Release of nucleotides by propionic acid, formic acid, or monochloroacetic acid shows the same pH dependence as acetate (Fig. 7). Since

the dissociation constants of formic, acetic, propionic, and monochloroacetic acids differ considerably from each other, the effect of pH on release of nucleotides seems to be due not to the effect of pH on the reagents added but rather to the effect of pH on a component of the nuclei with which acetate and the other substances react.

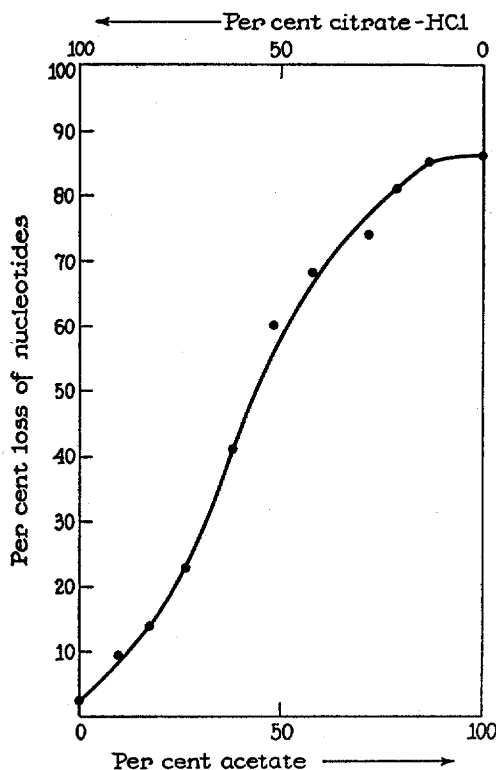


FIG. 6. Effect of varying acetate: citrate ratio on nucleotide retention in calf thymus sucrose nuclei.

To study the quantitative relationship between acetate and the removal of nucleotides from the nuclei, buffers were prepared containing a mixture of acetate and citrate at pH 4.5 in which the total molar concentration was kept constant but in which the acetate concentration was varied. It is evident from Fig. 6 that increasing concentration of acetate results in a corresponding increase in amount of nucleotides removed from the nuclei. In other experiments the amount and concentration of acetate were held constant but the amount of nuclear material was varied over a threefold range. It was found that the quantity of nucleotides released was proportional to the amount of

nuclear material present. Under these conditions, therefore, the concentration of acetate is the determining factor in releasing the nucleotides at the effective pH even when relative amounts of acetate and nuclei are varied. In further experiments on the acetate effect, we are planning to use $C^{14}$-labelled acetate to study the combination of acetate with nuclear constituents.

Our colleague, Dr. R. Lorente de Nó, has called our attention to some remarkable observations of his on the irreversible damage done to nerve fibers by a low concentration of acetate in a slightly acid medium (22). The analogy with the acetate effect on nuclei is suggestive.

It should be mentioned in passing that when nuclei are treated with acetate buffer at pH 4.5 in sucrose-$CaCl_2$, nucleotide triphosphates and diphosphates are largely converted to the monophosphate form. This dephosphorylation does not seem to have any relation to the specific effect of acetate in releasing nucleotides from the nuclei, for it was found that citrate buffer at the same pH (which does not release the nucleotides) also tends to dephosphorylate tri- and diphosphates.

It was found that when nucleotides are released from sucrose nuclei by acetate and other reagents, potassium in the nuclei is also released at the same time. All the potassium analyses to be mentioned were done by Dr. Shinji Itoh. Sucrose nuclei usually retain somewhat less than 50 per cent of the potassium present in the non-aqueous nuclei. This potassium is well preserved even after repeated washing of nuclei with cold sucrose-$CaCl_2$ solution (23). Fig. 7 includes the results of experiments in which the effects of a number of substances at various pHs on the retention of nucleotides and potassium were tested. One can see that there is a remarkable correlation between the retention of nucleotides and of potassium; over a wide range of pH as well as in the various kinds of reagents, both substances are in each instance released at the same time. It is not likely that the potassium released is combined with the nucleotides because the molar ratio of potassium to nucleotides removed is about twenty. The facts suggest rather that both substances are part of a common complex in the cell nuclei; acetate and other effective reagents disturb this complex in some way so that nucleotides and potassium are released.

We now have reasons to suppose that the nucleotides of the nucleus are held in some complex structure and that only such nucleotides are involved in the metabolism of the cell nucleus. The evidence for this is: (1) nucleotides are firmly held in the nucleus under a variety of conditions, and some special reagents such as acetate can release them together with potassium from the sucrose nuclei; (2) once nuclei lose their nucleotides, added nucleotides are not taken up; (3) only intranuclear nucleotides are phosphorylated—added nucleotides cannot be acted upon; (4) added AMP, ADP, or ATP have no effect on the synthesis of protein by isolated sucrose nuclei, whereas upon

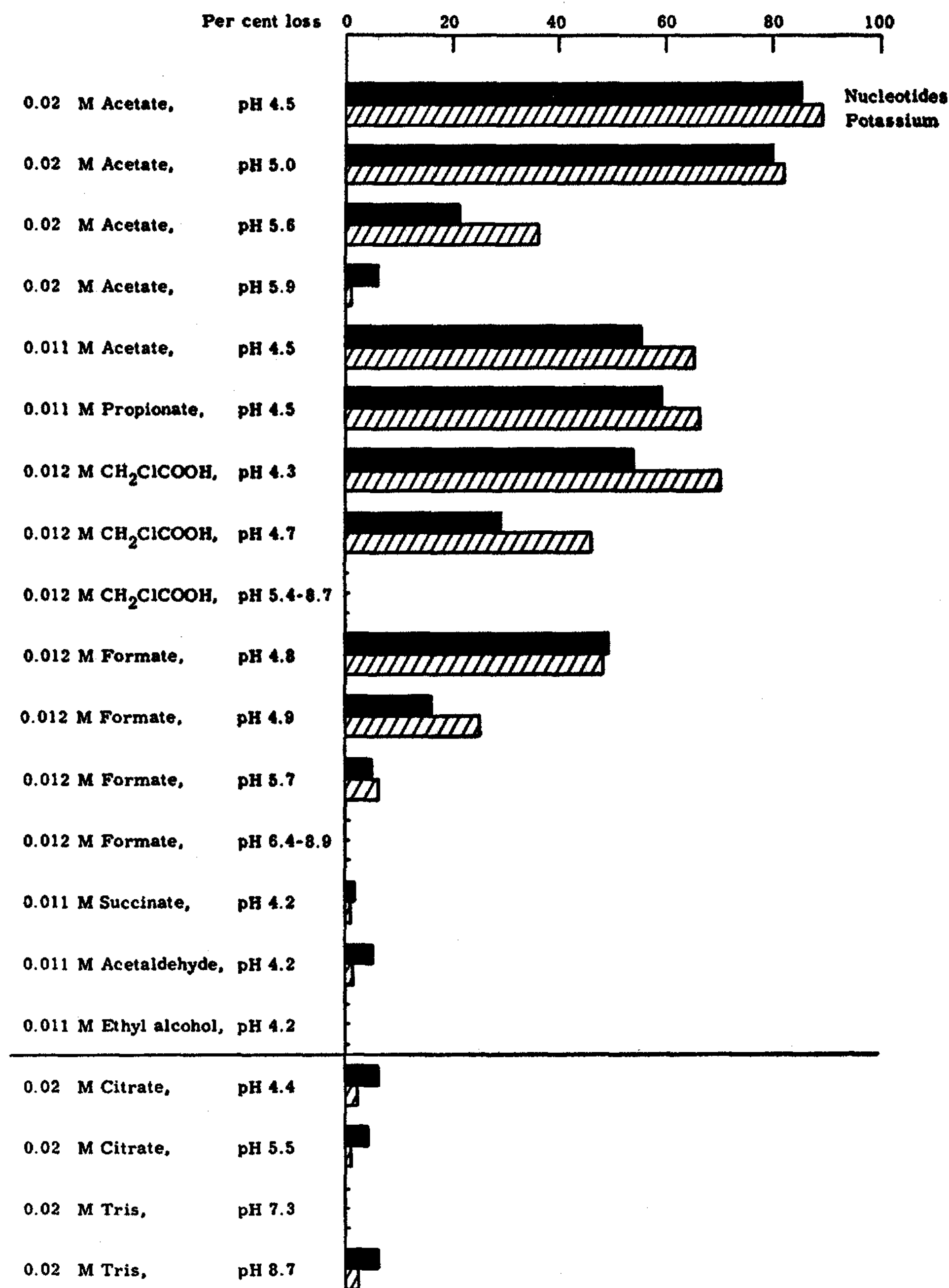


FIG. 7. Effect of some substances and varying pHs on nucleotide and K retention in calf thymus sucrose nuclei.

removal of nucleotides and potassium protein synthesis is seriously impaired; (5) under our experimental conditions intranuclear nucleotides, especially their triphosphates, are held to a considerable extent during the course of protein synthesis.

*Relation of Nucleotides and Potassium to Protein Synthesis in the Nucleus.*— The effect of removal of nucleotides and potassium on the process of protein synthesis in the nucleus has been studied. Since this involves exposing the nuclei to media in which the pH has been decreased, the effect of such exposure (without removal of nucleotides and potassium) was first investigated. After treating the nuclei with a sucrose solution containing an acid buffer (citrate or cacodylate) the nuclei were then washed with a sucrose–pH 6.7 phosphate mixture and the uptake of $C^{14}$-alanine into nuclear protein was measured under standard conditions. The way in which protein synthesis was reduced by exposure to acid media is shown Fig. 8 (the curve on the left). The release by acetate of nucleotides and potassium from nuclei occurs quite sharply as the pH is decreased and fortunately the point at which severe inactivation of protein synthesis by acid takes place. Exposure of nuclei to acetate prevents protein synthesis, when the nuclei are subsequently placed in a sucrose–pH 6.7 phosphate mixture, as shown in Fig. 8 (curve on the right). The release of nucleotides and potassium from nuclei by acetate and the destructive effect of acetate on protein synthesis in nuclei occur at a pH distinctly higher than that at which acidity *per se* greatly diminishes synthesis. These experiments demonstrate a correlation between the loss of nucleotides and potassium from nuclei and the impairment of protein synthesis in nuclei. Such a correlation is not, of course, a proof that the presence of nucleotides is required for protein synthesis.

Throughout this paper and the preceding one there have been comments on the role of sucrose in maintaining the integrity of the nucleus. It is well known that the microscopic appearance (in the light microscope) of an isolated nucleus is different when it is in a sucrose medium from what it is in a saline medium. If in a given preparation the medium is changed repeatedly from sucrose to saline and then back to sucrose the microscopic appearance of the nucleus changes with each change of medium; the alterations in microscopic appearance are readily reversed (24). The conditions within the nucleus which are preserved by sucrose solutions, and which have been significant for the present investigation, are different from those that have been recognized hitherto. The intranuclear conditions to which we now refer and the maintenance of which depends upon sucrose, when the nucleus is isolated, cannot be recognized by examination with the light microscope; and, furthermore, when these conditions within the nucleus are disturbed, restoration has not been possible. We are here dealing with what we must suppose are changes in the fine structure of the nucleus, changes which affect the metabolic activity of the nucleus.

That intranuclear conditions depend upon keeping isolated nuclei in a sucrose medium is shown by the following facts: (1) synthesis of protein stops if sucrose is removed and is not restored if the nuclei are again placed in a

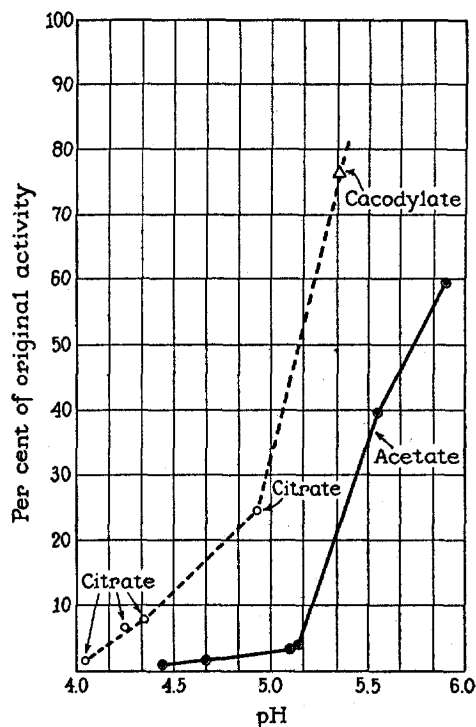


FIG. 8. Effect of the exposure of calf thymus sucrose nuclei to acidic buffer solutions on the subsequent synthesis of protein. Sucrose nuclei were exposed to the buffers in sucrose–$CaCl_2$ indicated in the figure. After 30 minutes, the nuclei were washed with 0.02 M sodium phosphate buffer in sucrose–$CaCl_2$ at pH 6.7, and tested for the incorporation of $C^{14}$-alanine into the nuclear proteins. Loss of nucleotides by the acidic buffer treatment from the nuclei was: in the citrate and cacodylate series, reading points on the curve from left to right, 6.1, 6.1, 5.1, 6.1, and 2.3 per cent of the control; in the acetate series, reading points on the curve from left to right, 90.5, 90.5, 84.0, 77.5, 26.5, and 9.3 per cent of the control. Abscissa represents the pH of the buffer solution measured after the treatment of nuclei.

sucrose medium; (2) mononucleotides are released when isolated nuclei are placed in a medium not containing sucrose and nucleotides are not again taken up by the nuclei when they are returned to a sucrose medium; (3) when sucrose nuclei are treated with DNAase they lose as much as 80 to 90 per cent of their DNA and yet only 15 per cent of their histone is released, although histone, a water-soluble protein, is attached to DNA in the nucleus; on the

other hand, when nuclei are in a saline medium (containing no sucrose) at the same time that DNA is removed by the action of DNAase about 50 per cent of the histone is released from the nuclei.

In our preparations the nuclei have been in isotonic sucrose, 0.25 M. If the sucrose concentration is raised to 0.50 M synthesis of protein no longer occurs, nor does it occur if the nuclei are returned to 0.25 M sucrose; apparently hypertonic sucrose causes an irreversible change in the fine structure of the nucleus. Retention of nucleotides in nuclei is not impaired by raising the sucrose concentration from 0.25 M to 0.50 M.

## EXPERIMENTAL

*Calf Thymus Sucrose Nuclei.*—Calf thymus glands were obtained as quickly as possible after the death of the animal, and transported to the laboratory in ice cold 0.25 M sucrose solution. Sucrose nuclei were prepared in 0.25 M sucrose containing a small amount of $CaCl_2$ as has been described before (1, 2). In earlier experiments, 0.25 M sucrose–0.002 M $CaCl_2$ was used. However, to prevent formation of nuclear gels, $Ca^{++}$ concentration has been increased up to 0.003 M in more recent experiments. All operations were done in a cold room at 2°C. The final volume of a nuclear preparation suspended in 0.25 M sucrose–$CaCl_2$ was such that the dry weight of the suspension was from 35 to 50 mg. per ml.

*Non-Aqueous Nuclei.*—Preparations from calf thymus, calf liver, and chicken erythrocytes as well as their corresponding whole tissues or cells used in this study were those described by Allfrey *et al.* (3), which had been kept at −20°C. until used.

*Trout Sperm Cells.*—Frozen dried brown trout sperm and rainbow trout sperm stored at −20°C. were used.

*Dry Weight.*—2 ml. of a sucrose nuclear suspension was centrifuged at 4,000 R.P.M. for 5 minutes in the cold. The sediment was suspended in 7 ml. of 88 per cent alcohol, and kept in a water bath at 60°C. for 5 to 10 minutes. After centrifugation, the sediment was incubated in the same way as before with 88 per cent alcohol twice, and 95 per cent alcohol twice. It was then washed with 7 ml. of ether twice, and dried at 110°C. All centrifugations were done at room temperature. It is necessary to wash the sucrose nuclei with hot 88 per cent alcohol for the complete removal of the sucrose present. Weighed samples of fresh tissue and non-aqueous preparations were treated with 95 per cent alcohol three times, and with ether twice. They were then dried at 110°C. and weighed again.

*Preparation of an Acid-Soluble Extract for the Chromatographic Fractionation of Nucleotides.*—All operations were carried out in a cold room at 2°C. To 8 volumes of nuclear or tissue suspension, 2 volumes of 10 per cent perchloric acid were added, and stirred for about 10 minutes. The precipitated protein was sedimented at 5,000 R.P.M. for 5 minutes, and extracted again with 5 volumes of 2 per cent perchloric acid. The combined supernatant was brought to pH 6 to 7 by careful dropwise addition of 6 N KOH. The precipitate of $KClO_4$ was removed by centrifugation. Part of the supernatant was used for an extinction measurement at 260 m$\mu$. A large aliquot was used for the chromatographic analysis of nucleotides.

*Chromatographic Fractionation of Nucleotides.*—The "formic acid system" of

dowex 1–formate of Hurlbert *et al.* (4) was used throughout this investigation. The elution system was adapted for the fractionation of relatively small amounts of samples by using a 100 ml. reservoir, a 50 ml. mixing flask, and a 0.6 × 20 cm. dowex 1–formate resin column. Adsorbed nucleotides were eluted with a continuously increasing concentration of eluant, that is, first with 100 ml. of 4 N formic acid, followed by successive 100 ml. volumes of 0.2 M ammonium formate in 4 N formic acid, 0.4 M ammonium formate in 4 N formic acid, and 1 M ammonium formate in 4 N formic acid. Samples of effluent containing 1.3 ml. for a 15 minute flow were collected automatically. The extinction at 260 m$\mu$ was measured for each tube to determine the quantity of nucleotide present. The extinction at 275 m$\mu$ was also measured for an approximate characterization of each peak. Each nucleotide peak was identified by its location on the dowex 1–formate chromatograms, and also after lyophilization the material in each peak was identified by its ultraviolet spectrum, orcinol reaction, diphenylamine reaction, etc. (*cf.* Hurlbert *et al.* (4)).

*Postmortem Changes of Nucleotides in Rat Thymus Tissue.*—The rats were kindly given to us by Dr. John B. Nelson of The Rockefeller Institute. Twelve individuals, age 8 to 9 weeks, were killed by decapitation, and their thymus glands were removed. Some of them were immersed in liquid nitrogen immediately upon removal, and lyophilized. Dried thymus corresponding to 1 gm. of wet weight was ground in a mortar with 10 ml. of cold 2 per cent perchloric acid. The acid-soluble extract was prepared as described before. 1 gm. samples of freshly excised thymus tissue were incubated in glass tubes in a water bath at 30°C. for 5, 10, and 20 minutes. After incubation, 10 ml. of cold 2 per cent perchloric acid was added to each sample and an acid-soluble extract was prepared. All samples were chromatographed on a dowex 1–formate column. The average weight of the thymus from rats of the ages used was 0.33 gm.

*Phosphorylation in Isolated Nuclei.*—A sucrose nuclear preparation was made quickly (in 26 minutes) from 50 gm. of calf thymus, and finally suspended in 35 ml. sucrose–$CaCl_2$. To a 15 ml. aliquot of the nuclear suspension 4 ml. of 10 per cent perchloric acid was immediately added and an acid-soluble extract was prepared. Another 15 ml. sample was stirred gently with a mechanical stirrer for 50 minutes at 2°C. At the end of this time, another acid-soluble extract was made. Both samples were chromatographed.

*Phosphorylation in a Calf Thymus Homogenate.*—140 gm. of calf thymus was minced and homogenized with 630 ml. of 0.25 M sucrose–0.003 M $CaCl_2$ in a low speed blendor (1,300 R.P.M.) for 4 minutes. The homogenate was strained through gauze and then through flannelette. The volume of the strained fluid was 580 ml. A perchloric acid extract was immediately prepared by adding 60 ml. of 10 per cent perchloric acid to 250 ml. of this homogenate. Another 250 ml. sample was allowed to stand for 95 minutes at 4°C. with gentle mechanical stirring, and was then centrifuged for 7 minutes at 3,000 R.P.M., the supernatant being the cytoplasmic fraction. This was centrifuged again to remove the nuclei still present. The nuclear sediment from the first centrifuging was washed once with cold sucrose–$CaCl_2$. The supernatant was combined with the cytoplasmic fraction. Acid-soluble extracts were prepared from both nuclear and cytoplasmic fractions. All acid-soluble extracts were analyzed chromatographically for nucleotides.

*Phosphorylation in Presence of Added AMP.*—Calf thymus sucrose nuclei were prepared quickly and suspended in 35 ml. of sucrose–$CaCl_2$ solution. To 10 ml. aliquots were added: (1) 1.63 ml. 0.25 M sucrose containing 5 mg. AMP (pH 6.8); (2) 1.84 ml. 0.025 M sodium phosphate buffer at pH 6.8 in 0.25 M sucrose containing 5 mg. AMP; (3) 2.0 ml. 0.25 M sucrose. They were then stirred gently for 120 minutes at 20°C. The acid-soluble extract of each sample was analyzed for nucleotides on a dowex 1–formate column.

*Inorganic Phosphate.*—Analyses of inorganic phosphate in the sucrose nuclei present a difficulty because of the presence in the 2 per cent perchloric acid extract of lysine-rich histones which form a heavy precipitate with ammonium molybdate. In this study both of the following ways were adopted.

(*a*) 10 ml. of a cold 2 per cent perchloric acid extract of sucrose nuclei was carefully neutralized with 6 N KOH, and insoluble $KClO_4$ was removed by centrifugation. The clear supernatant was then dialyzed in a cellophane bag against distilled water overnight in a cold room. The volume of outside plus inside fluid was 20 ml. Inorganic phosphate was determined on an aliquot of outside fluid.

(*b*) 20 ml. of cold 2 per cent perchloric acid extract of sucrose nuclei was neutralized with 6 N KOH, followed by the centrifugation of the $KClO_4$ precipitate in the cold. The supernatant was then chromatographed on a dowex 1–formate column. A peak of inorganic phosphate was lyophilized. The dry material was dissolved in 10 ml. of water. Inorganic phosphate was analyzed on an aliquot of this solution.

*Effect of Inhibitors on Nuclear Phosphorylation.*— Effect of NaCN, 2,4-dinitrophenol, sodium azide, dicumarol, Janus green B (National Aniline Division, CI No. 133), methylene blue (Grübler, Leipzig), antimycin A.—About 150 gm. of calf thymus tissue was grossly minced. 50 gm. of this was further finely minced in a sucrose–$CaCl_2$ solution containing an appropriate amount of one of the above mentioned inhibitors. Nuclei were kept in the presence of inhibitor throughout their isolation. The isolated nuclei were suspended in 15 to 20 ml. of sucrose–$CaCl_2$ containing an inhibitor, and allowed to stand for 60 minutes. The concentration of each inhibitor was kept constant throughout the experiment. Control nuclei were prepared in parallel from 50 gm. of minced tissue and treated in the same way in the absence of inhibitor. Acid-soluble extracts were chromatographically analyzed for nucleotides. The extent of inhibition of phosphorylation of nuclear AMP was calculated from the per cent of the total extinction at 260 m$\mu$ of ADP and ATP (ADP as 1, and ATP as 2) found in the experimental and control series. The per cent of the total extinction at 260 m$\mu$ of ADP and ATP at the beginning of the experiment was estimated on the whole tissue, because it has been shown by the analyses of non-aqueous preparations that the relative concentration of AMP, ADP, and ATP in whole tissues and in the nuclear fraction is the same.

*Effect of Buffers at Different pHs, and of Some Reagents on the Retention of Nucleotides and Potassium in Sucrose Nuclei.*—To 1 ml. of sucrose nuclei 9 ml. of 0.022 M buffer solutions in 0.25 M sucrose–0.003 M $CaCl_2$ was added, stirred, and allowed to stand for 30 minutes at 2°C. The suspension was centrifuged at 5,000 R.P.M. for 5 minutes. As control 9 ml. of sucrose–$CaCl_2$ without buffer was used. The pH of each supernatant while cold was determined with a glass electrode. 1 ml. of 10 per cent perchloric acid was added to 4 ml. of supernatant to precipitate protein if present.

The extinction at 260 mμ was read on the clear perchloric acid supernatant, and also on the 2 per cent perchloric acid supernatant obtained directly from 1 ml. of original nuclei. Acetaldehyde, alcohol, acetone, succinic acid, propionic acid, and monochloroacetic acid were used in a desired concentration by mixing with citrate buffer–sucrose–$CaCl_2$. The pH of each solution was adjusted with 1 N NaOH or HCl before use. The effects of these substances were tested under the conditions mentioned above.

Potassium analyses were kindly performed for us by means of a flame photometer by Dr. Shinji Itoh on the residue obtained when the sucrose suspensions of nuclei were centrifuged in the experiments described above.

*Effect of the Removal of Nucleotides and Potassium by Acetate from Sucrose Nuclei on the Synthesis of Protein.*—Each 10 ml. sample of calf thymus sucrose nuclear suspension was centrifuged down at 3,000 R.P.M. for 7 minutes at 2°C. The sediments were then taken up to 25 ml., each with one of the following solutions: *control series* (1) 0.25 M sucrose–0.003 M $CaCl_2$, (2) citrate buffer pH 4.0, (3) citrate buffer pH 4.2, (4) citrate buffer pH 4.5, (5) citrate buffer pH 5.0, (6) cacodylate buffer pH 5.4; *acetate series*- acetate buffer (1) pH 4.0, (2) pH 4.45, (3) pH 4.75, (4) pH 4.95, (5) pH 5.3, (6) pH 5.7. All buffers were in 0.25 M sucrose–0.003 M $CaCl_2$ at the concentration of 0.02 M. Each suspension was then allowed to stand for 30 minutes in the cold. After centrifugation at 3,000 R.P.M. for 7 minutes, the pH and the extinction at 260 mμ were determined on each supernatant (for procedure see preceding section). The residue was washed with 25 ml. of 0.02 M sodium phosphate buffer at pH 6.7 in sucrose–$CaCl_2$. 1 ml. of each nuclear suspension was used for the $C^{14}$-alanine incorporation experiments as described in the previous paper (1). The extent of $C^{14}$-alanine incorporation by each nuclear preparation was expressed as "per cent of original activity," taking the preparation (1) in the control series as 100.

## SUMMARY

1. It has been demonstrated by ion exchange chromatography that the cell nucleus contains mononucleotides of adenine, guanine, cytosine, uracil, together with diphosphopyridine nucleotide, and several uridine diphosphate derivatives; the adenine nucleotides predominating in amount. Nucleotide components in the cell nucleus are in close agreement both quantitatively and qualitatively with those found in the cytoplasm.

2. In calf thymus sucrose nuclei, nucleotide monophosphates can be phosphorylated to the energy-rich triphosphate form *without* participation of cytoplasmic components. As to the nature of the phosphorylation, it has been shown that there exist certain differences as well as resemblances between nuclei and mitochondria. A distinctive feature of nuclear phosphorylation is that only intranuclear monophosphates seem to be phosphorylated. The process is completely inhibited by cyanide, azide, and dinitrophenol. However, certain reagents which block oxidative phosphorylation of mitochondria, namely dicumarol, Janus green B, methylene blue, and calcium ions, have no effect on phosphorylation within the nucleus.

3. The bulk of mononucleotides is preserved within thymus nuclei after their isolation in sucrose. Nucleotides are surprisingly well retained by nuclei in a sucrose medium whether or not electrolytes are present and in buffers ranging from pH 3 to 10; under all conditions sucrose is required for retention.

4. Dilute acetate in sucrose releases nucleotides from the nucleus below pH 5.1. As to the effective pH of acetate, there is a sharp boundary between pH 5.1 and pH 5.9. At pH 5.9, and above, acetate does not remove nucleotides from the nucleus. The effects of propionate, formate, and monochloroacetate on the nuclei are the same as that of acetate.

5. When nuclei are exposed to a wide variety of conditions a close correlation is found between the retention in the nucleus of nucleotides and of potassium. This suggests that both substances are part of a common complex in the cell nucleus.

6. It has been shown that upon removal of nucleotides and potassium from calf thymus sucrose nuclei by acetate, the ability to incorporate $C^{14}$-alanine into nuclear protein is greatly impaired.

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