PROTEIN SYNTHESIS IN ISOLATED CELL NUCLEI

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The present communication deals with the ability of isolated nuclei to incorporate isotopically labelled amino acids into their proteins. It will be shown that the deoxyribonucleic acid of the nucleus plays a role in this incorporation; that protein synthesis virtually ceases when the DNA is removed from the nucleus, and that the uptake of amino acids resumes when the DNA is restored.

This report also deals with the conditions necessary for amino acid incorporation, with the effects of various inhibitors, and with the role of nucleic acid components in this aspect of protein synthesis. Preliminary accounts of some of these experiments have been published elsewhere (1, 2).

Some Properties of Isolated Thymus Nuclei.—Most of the experiments to be described were performed on nuclei isolated from calf thymus tissue in 0.25 M sucrose solution containing a small amount of calcium chloride. The procedure is rapid and simple, and it provides nuclei of high purity in good yield.

Since some form of standardization is essential for all work on isolated cell components, thymus nuclei isolated in sucrose have been compared with those isolated in non-aqueous media (3, 4). The purpose of this comparison is to test whether the aqueous isolation procedure extracts water-soluble nuclear components, or whether it leads to excessive contamination with the water-soluble proteins of the cytoplasm. Many such comparisons have shown that thymus "sucrose" nuclei are the equivalent of the standard "non-aqueous" nuclei in many respects. Their DNA content is the same (2.5 per cent DNA-P), as is their over-all protein composition and enzymic constitution (4).

When preparations of thymus nuclei isolated in sucrose solution are examined under the light microscope, either stained or unstained, they seem to be a beautiful preparation. One observes great numbers of free nuclei, a few whole cells, and occasional red cells. Staining reveals occasional strands of cytoplasm attached to some of the nuclei. The over-all extent of this cytoplasmic contamination can be estimated chemically [*e.g.* by nucleic acid analyses] and the conclusion was reached that these nuclei are better than 90 per cent pure.

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Since many intact thymocytes have only a thin halo or crescent of cytoplasm, the possibility was considered that many objects believed to be free nuclei under the light microscope might be intact cells with scanty or negligible amounts of cytoplasm. To investigate this possibility two preparations

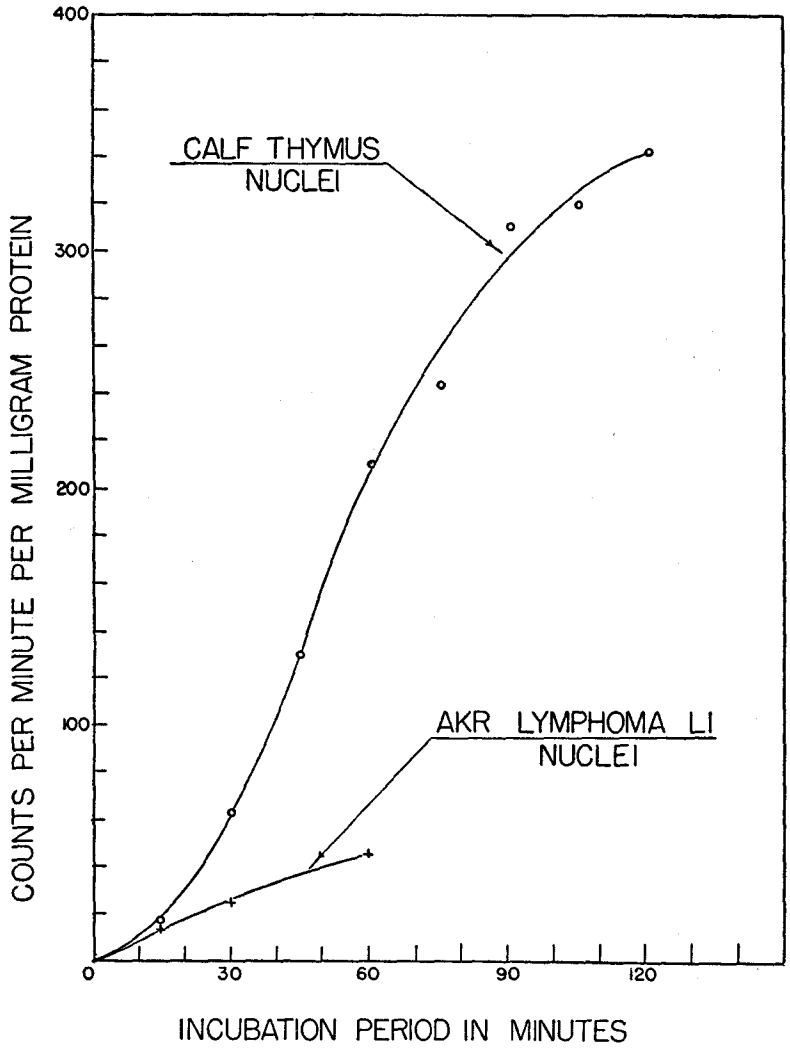

FIG. 1. The time course of alanine-1-C^{14} incorporation into the proteins of isolated nuclei. The specific activity of the total nuclear protein is plotted against the time of incubation of the nuclear suspension. The upper curve shows the incorporation into thymus nuclei. The lower curve shows the rate of alanine-C^{14} uptake by nuclei isolated from an AKR lymphoma.

were examined under the electron microscope. (This was made possible through the generous cooperation of Dr. M. Watson and Dr. G. Palade of The Rockefeller Institute.) Electron microscopy makes it evident that there is some cytoplasmic contamination, but this is also evident in stained preparations under the light microscope. The advantage of the electron microscope in these studies is the ease of detection of small thymocytes. Cell counts on random fields selected from the two preparations gave 45 cells per thousand nuclei in the

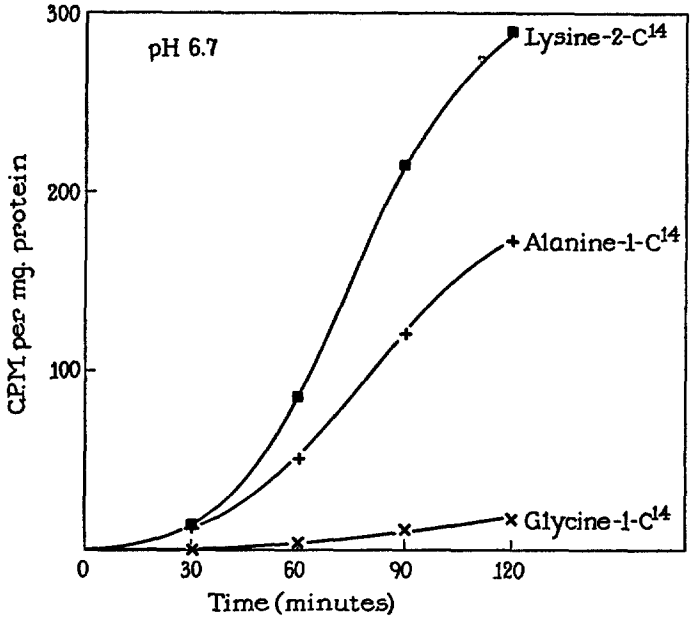

FIG. 2. The time course of incorporation of alanine-1-C^{14}, glycine-1-C^{14}, and lysine-2-C^{14} into the proteins of isolated thymus nuclei. In this plot the data have been "normalized" to show the relative uptakes for equivalent amounts of the different amino acids, when each has the same specific activity in millicuries per millimol.

first preparation and 77 per thousand in the second. Even more convincing evidence for the absence of appreciable whole cell contamination was obtained in studying the effect of deoxyribonuclease. These experiments are described in detail below.

Amino Acid Incorporation into the Proteins of Isolated Nuclei.—When thymus nuclei are suspended in a buffered sucrose medium and incubated at 37°C., in the presence of isotopically labelled amino acids, there is a rapid and considerable incorporation of the isotope into the proteins of the nucleus. Fig. 1 shows the time course of incorporation of alanine-1-C^{14} into the mixed proteins of isolated calf thymus nuclei. The lower curve shows the incorporation

observed into lymphoma nuclei isolated by a similar procedure. (The lymphoma tissue was generously supplied by Dr. John Kidd of Cornell University Medical College.) After an initial "lag" period of about 10 to 15 minutes, the

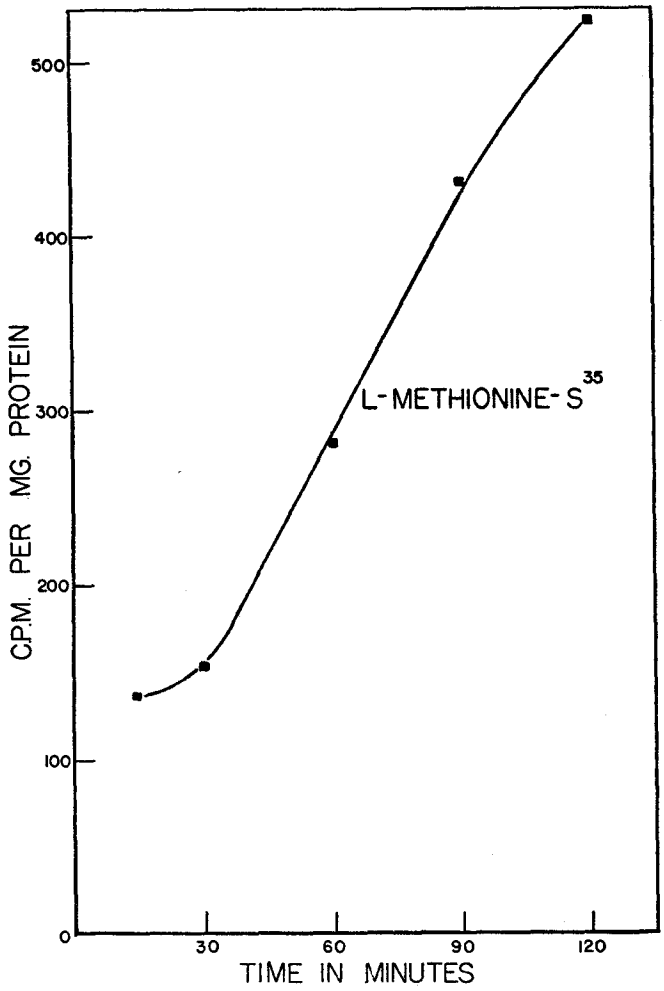

FIG. 3. The time course of methionine-S^{35} incorporation into the proteins of isolated thymus nuclei.

C^{14} uptake proceeds linearly for about 90 minutes and then begins to slowly taper off.

Because a few whole cells are inevitably present in preparations of thymus nuclei, the possibility was considered that the observed amino acid uptake was due to the cells and not to the nuclei. To test this possibility a radioautograph was made of a smear of the nuclear suspension after incubation

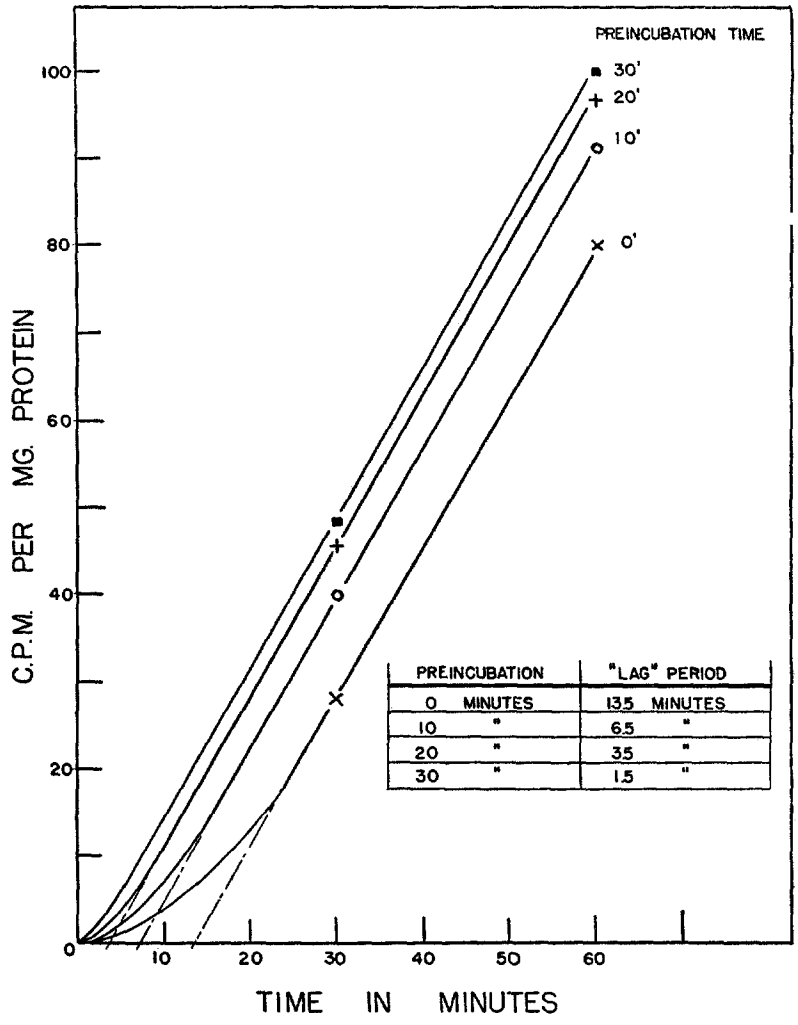

FIG. 4. The effect of preincubation of thymus nuclei upon the subsequent incorporation of alanine-1-C^{14}. The nuclear suspension was preincubated for 10, 20, or 30 minutes before adding the isotopic alanine. The time course of C^{14} incorporation by such preincubated nuclei is compared with that of control nuclei which received the isotope at time zero. Note that preincubation diminishes the lag period without affecting the subsequent rate of C^{14}-alanine incorporation.

with C^{14}-alanine and acid fixation to remove the soluble amino acid. The distribution of silver grains produced in an overlaying photographic emulsion was then examined under the microscope, and it was observed that all components of the suspension were radioactive. The uptake was not simply due

to the few cells present in the smear. Similar work in other laboratories also makes it plain that individual nuclei incorporate the radioactive amino acid (5).

Figs. 2 and 3 show the time course of alanine-1-C^{14}, glycine-1-C^{14}, lysine-2-C^{14}, and methionine-S^{35} incorporations. In these and in most of the experiments to be described below, the uptake was followed by measuring the radioactivity of the total mixed proteins of the nucleus. The results are expressed

TABLE I

Effect of Anoxia and of Several Inhibitors upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	Degree of inhibition
		C.P.M./mg.	*per cent*
1. Nuclei in O_2	Glycine-1-C^{14}	71	—
" " N_2	"	15	80
2. Nuclei in O_2	DL-Alanine-1-C^{14}	72	—
" " N_2	"	10	85
3. Control nuclei	"	72	—
Nuclei in 2×10^{-4} M dinitrophenol	"	11	84
4. Control nuclei	"	131	—
Nuclei in 2×10^{-4} M Janus green B	"	3	97
5. Control nuclei	"	54	—
Nuclei in 1×10^{-3} M NaCN	"	13	76
" " " " NaN_3	"	5	91
Nuclei + 0.05 mg. dicumarol per ml.	"	8	85
" + 1 μg. antimycin A " "	"	6	89

as counts per minute per milligram of protein. The protein was prepared by first extensively washing the nuclei with trichloroacetic acid (TCA), removing the nucleic acids with hot TCA, and finally removing the lipides with warm ethanol, ethanol-ether-chloroform mixtures, ether, and acetone. The protein residue was then homogenized in acetone and filtered off on filter paper planchets. Radioactivity was determined using a Geiger-Müller tube and scaling circuit, and the measurements were subsequently corrected for self-absorption (6).

To test whether the radioactivity measured was actually in the protein and not merely adsorbed or bound by ester linkages, the protein was treated with alkali and with ninhydrin. In the case of incorporated alanine-1-C^{14}, more than 94 per cent of the activity remained after treatment with 0.25 N NaOH for 2 hours, and more than 89 per cent remained after reaction with

ninhydrin. 76 per cent of the incorporated methionine-S^{35} was stable to alkali. When the different proteins of the nucleus are fractionated before counting, the label remains throughout the fractionation and can be detected in such well defined nuclear components as the "arginine-rich-" and "lysine-rich" histones.

The nature of the lag in the uptake of radioactive alanine into the proteins of the nucleus was investigated by experiments which are summarized in Fig. 4. Nuclei were preincubated at 37° for 10, 20, or 30 minutes before adding the isotopic alanine. The time course of C^{14} incorporation by such preincubated

TABLE II

Effect of Fluoride and of Added Metabolites upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of protein
		c.p.m./mg.
1. Control nuclei	DL-Alanine-1-C^{14}	62
Nuclei in 1×10^{-3} M Na F	"	57
2. Nuclei without added metabolite	"	40
" + 40 μM glucose	"	44
" " " " fructose	"	40
3. Nuclei without added metabolite	Glycine-1-C^{14}	86
" + 40 μM glucose	"	99
" " " " α-ketoglutarate	"	109

nuclei was compared with that of control nuclei which had received the isotope at time zero. It is evident that preincubation diminishes the lag period without affecting the subsequent rate of C^{14}-alanine incorporation. These findings suggest that an activation of some sort occurs during preincubation. Further evidence for such activation will be presented below in connection with the inhibitory effects of benzimidazole derivatives. [The possibility was also considered that the lag in C^{14}-alanine uptake might be due to the existence of a "fixed pool" of unlabelled alanine in the nuclei, a pool which must be depleted before isotopic alanine is taken up. This would be analogous to the interesting situation described by Cowie and Walton (7) of fixed amino acid pools in yeast. There is, however, a fundamental distinction between the two systems. In their experiments labelled amino acids in the pool went directly to protein, and the rate of this incorporation was not affected by adding C^{12}-amino acids to the medium. Similar experiments with isolated thymus nuclei have shown that exogenous, unlabelled L-alanine is in rapid and free exchange with the C^{14}-alanine of the pool.]

There are a number of experiments now to be described which deal with

the conditions necessary for amino acid incorporation, and with the inhibition of nuclear protein synthesis.

The uptake of amino acids is an aerobic phenomenon which does not occur in a nitrogen atmosphere and which is inhibited by a number of substances

TABLE III

Effect of Chloramphenicol, Cortisone, and Amino Acid Antagonists upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	Change in C^{14} or S^{35} uptake
		c.p.m./mg.	*per cent*
1. Control nuclei	DL-Alanine-1-C^{14}	97	—
Nuclei in 3.2×10^{-4} M chloramphenicol	"	97	0
2. Control nuclei	Glycine-1-C^{14}	21	—
Nuclei in 1.6×10^{-4} M chloramphenicol	"	19	−10
3. Control nuclei	L-Methionine-S^{35}	137	—
Nuclei in 1.6×10^{-4} M chloramphenicol	"	136	0
4. Control nuclei	DL-Alanine-1-C^{14}	43	—
Nuclei in 1×10^{-3} M *p*-fluorophenylalanine	"	37	−14
5. Control nuclei	"	37	—
Nuclei in 5×10^{-3} M *p*-fluorophenylalanine	"	31	−15
6. Control nuclei	"	65	—
Nuclei in 1.5×10^{-4} M ethionine	"	56	−14
" " " " 10^{-3} " "	"	51	−21
7. Control nuclei	DL-Lysine-2-C^{14}	23	—
Nuclei in 1.5×10^{-4} M ethionine	"	22	−4
" " " " 10^{-3} " "	"	22	−4
8. Control nuclei	L-Methionine-S^{35}	221	—
Nuclei in 1.5×10^{-4} M ethionine	"	215	−3
" " " " 10^{-3} " "	"	194	−12
9. Control nuclei	DL-Alanine-1-C^{14}	217	—
Nuclei + 0.063 mg. cortisone	"	138	−36
" " 0.125 " "	"	122	−54

which are known to block oxidative phosphorylation (Table I). Cyanide, azide, dinitrophenol, Janus green b, dicumarol, and antimycin A all inhibit alanine uptake. However, methylene blue, a substance which inhibits oxidative phosphorylation in mitochondria, has only a small effect on nuclear isotope

incorporation. The nature of the oxidative phosphorylation in preparations of isolated thymus nuclei is discussed in detail in the following paper.

Adding metabolites, such as glucose, fructose, or α-ketoglutarate to the nuclear suspension has little effect on the incorporation of amino acids (Table II). Added glucose may at times raise the alanine uptake by about 10 per cent. An attempt to block glycolysis by the addition of fluoride did not result in inhibition of alanine incorporation.

It was shown by Gale and Folkes that chloramphenicol blocks protein synthesis in bacteria (8). This substance has little effect on glycine-1-C^{14}, alanine-1-C^{14}, or methionine-S^{35} uptake by isolated nuclei (Table III). The

TABLE IV

Effect of Benzimidazole Derivatives on Alanine-1-C^{14} Uptake by Isolated Nuclei

Derivative tested		Amount	Specific activity of nuclear protein	Inhibition of uptake	Relative inhibition of virus multiplication*
		mg.	*C·P·M./mg.*	*per cent*	
Control		—	50	—	—
5,6-Dichlorobenzimidazole	1-β-D Arabinopyranoside	0.1	45	10	3.1
"	1-β-D-Ribopyranoside	0.1	36	28	15
"	1-β-D-Ribofuranoside	0.1	26	48	92
4,5,6-Trichlorobenzimidazole	1-α-D-Ribofuranoside	0.01	46	8	165
"	1-β-D-Ribofuranoside	0.01	36	28	760

* Work of Tamm *et al.* (11–13).

table also summarizes some experiments using *p*-fluorophenylalanine, an amino acid antagonist, which was shown by Halvorson and Spiegelman to block protein synthesis in bacteria (9). We were unable to test this compound at the higher concentrations used in Halvorson's experiments, but at 5×10^{-3} M it inhibited alanine uptake by only 15 per cent. Similarly, added ethionine (1.5×10^{-4} M) had no effect on the incorporation of methionine-S^{35} or lysine-2-C^{14}, and it reduced alanine uptake by only 14 per cent.

In considering possible inhibitors of nuclear protein synthesis, our attention was directed to the remarkable observation that repeated injections of cortisone lead to involution of the thymus gland (10). When tested on thymus nuclei, *in vitro*, small amounts of cortisone lead to greatly reduced alanine-C^{14} uptakes (Table III).

Among the inhibitors tested, a series of substituted benzimidazoles was of greatest interest. Typical of this class of compounds is the substance 5,6-

dichloro-β-D-ribofuranosylbenzimidazole (DRB). This structural analog of a purine riboside was found by Tamm *et al.* to retard influenza virus multiplication in the mouse and in tissue culture (11–13). When tested on isolated thymus nuclei it effectively inhibits the uptake of alanine-1-C^{14}. Tests of this

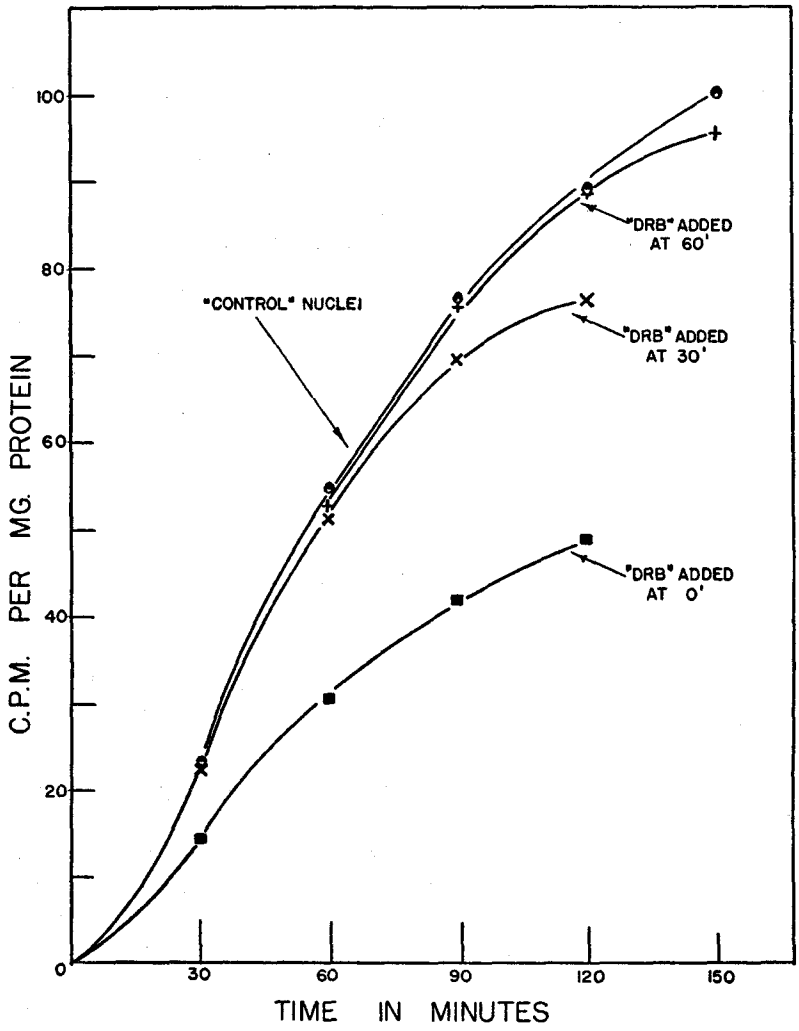

FIG. 5. The effect of 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB) on alanine-1-C^{14} incorporation into the proteins of isolated thymus nuclei. The time course of C^{14} incorporation in the presence of DRB is compared with that observed in control nuclei (upper curve). Note that DRB added together with the isotopic alanine (at time zero) is an effective inhibitor, whereas DRB added 30 or 60 minutes later is far less effective.

and similar compounds (synthesized by C. Shunk and K. Folkers of the Merck Research Laboratories) are summarized in Table IV. It is of special interest that the inhibitions observed in the isolated nuclei closely parallel those found in tests of viral growth. The ribosyl benzimidazole is an effective inhibitor while the corresponding arabinose derivative is not. Effective inhibition also requires that the ribose exist in the furanose, not the pyranose form, and

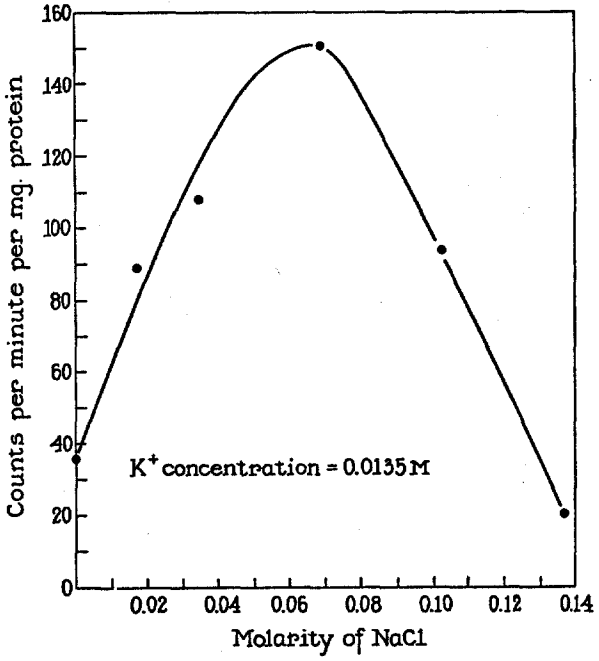

FIG. 6. The effect of varying the sodium ion concentration of the incubation medium on the ability of thymus nuclei to incorporate glycine-1-C^{14}. The specific activity of the nuclear protein after 60 minutes' incubation of the nuclei in the presence of glycine-C^{14} is plotted against the sodium concentration of the medium.

that the glycosidic linkage between the sugar and the benzimidazole ring should have the β-, and not the α-, configuration.

A remarkable feature of these inhibitors in the viral growth system is the limitation of their time of action. They effectively retard virus multiplication only if they are present at the time of infection or shortly afterward, and much of their effectiveness is lost if they are administered several hours after infection has occurred. This limitation on the time of action is also evident when benzimidazole derivatives are tested on isolated thymus nuclei. The experiments summarized in Fig. 5 show that when 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB) is added together with the isotopic alanine, the

uptake is inhibited from the outset, and for the duration of the experiment. However, when the DRB is added 30 or 60 minutes after adding the C^{14}-alanine there is no appreciable inhibition of the amino acid uptake. The findings suggest that DRB interferes with a preliminary "activation" of nuclear pro-

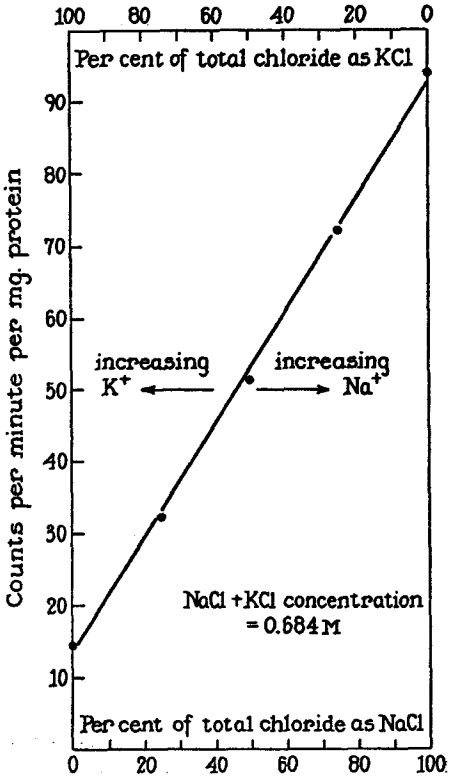

FIG. 7. The effect of varying sodium/potassium ratio on glycine-1-C^{14} incorporation by isolated thymus nuclei. The specific activity of the nuclear protein after 60 minutes' incubation is plotted against the ratio of sodium to potassium ions in the medium, the total salt concentration being held constant.

tein synthesis, and that once this activation has occurred the inhibitor is no longer effective. This agrees with the conclusions drawn previously from the study of the lag phase in alanine-1-C^{14} incorporation. Evidence is presented below to suggest that DRB inhibits the synthesis of protein by interfering with a preliminary synthesis of ribonucleic acid.

One of the most striking effects on nuclear amino acid incorporation is that produced by sodium ions. Nuclei isolated in sucrose need a sodium supplement in order to incorporate amino acids actively. Fig. 6 shows the effect of varied

sodium concentration on the level of glycine-1-C^{14} uptake. There is a well defined maximum at about 0.07 M under our test conditions. The sodium requirement seems to be specific. An attempt was made to substitute all or part of the sodium in the medium with an equivalent amount of potassium. In these experiments the total salt concentration was kept constant and the

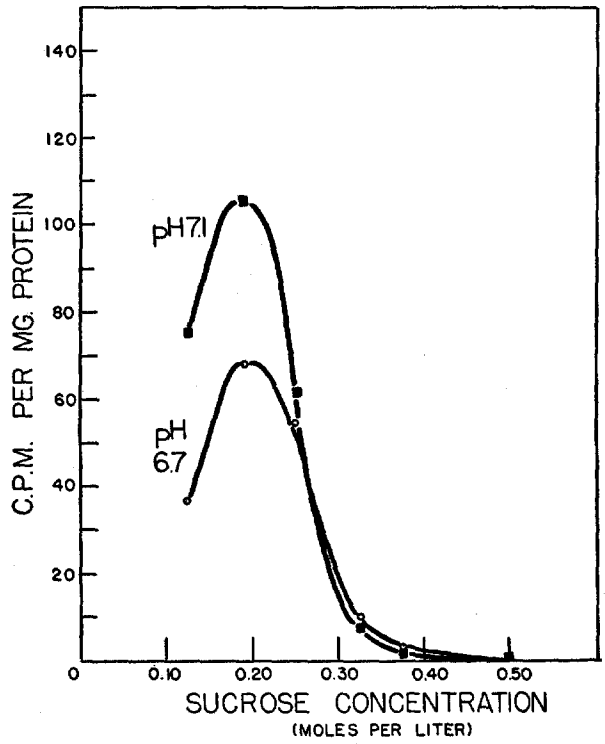

FIG. 8. The effect of varying the sucrose concentration of the medium upon the ability of thymus nuclei to incorporate alanine-1-C^{14}. The specific activity of the nuclear protein after 60 minutes' incubation is plotted against the sucrose concentration of the incubation medium.

ratio NaCl/KCl was varied. When all the sodium is replaced by potassium the uptake falls to 15 per cent of the optimal value. Increasing the sodium to potassium ratio gives a corresponding increase in the amount of amino acid incorporated (Fig. 7). When the sodium concentration is optimal, the addition of small amounts of potassium (chloride) does not influence the uptake.

The extreme dependence of the nucleus' capacity to incorporate amino acids upon sodium ion concentration makes it necessary to control rather rigorously the sodium level of the incubation medium. This introduces a related problem,

namely the osmotic balance between nuclear structures and the suspending medium. It was soon found that nuclei exposed to high sucrose concentrations lost their ability to incorporate amino acids into protein. A more detailed study of this dependence of uptake upon sucrose concentration is presented in Fig. 8. The peak of activity under our test conditions occurs at about 0.20 M. The medium also contains sodium phosphate buffer (0.025 M), glucose (0.02 M), and NaCl (0.03 M). Although the effects described bear some resemblance to the osmotic properties of semipermeable membranes, it should be pointed out that thymus nuclei are not enveloped by an intact, semipermeable membrane, and that they are freely permeable to large molecules, such as ribonuclease, deoxyribonuclease, histones, protamines, and basic dyes. The marked effects of sucrose concentration upon nuclear activity suggest that the fine structure necessary for nuclear protein synthesis is in osmotic balance with its environment, and that nuclear function can vary with that balance. This is reminiscent of experiments on the extended and contracted states of interphase chromosomes (14).

Although it is a simple matter to control the sucrose concentration and sodium level of the incubation medium, there are other factors which influence nuclear amino acid uptake which are not readily subject to control. In working with tissue obtained from commercial packers one finds variations in the age of the thymus, and in the sex and physiological condition of the animal. Weather and feeding, too, can vary the synthetic activity of the nucleus in ways which cannot be predicted or controlled. Thus the actual amount of amino acid incorporated in a given time varies from one nuclear preparation to another, and it is necessary to run daily controls when comparisons are being made.

The synthetic activity of the isolated nucleus can be readily destroyed by heating, by freezing and thawing, or by breaking in a high-speed blendor. Nuclei removed from sucrose to salt solutions, or washed with 0.1 M phosphate buffer prior to incubation in a sucrose medium, lose their ability to incorporate amino acids. Nuclei stored at 2°C. gradually lose their synthetic activity. The rate of decline in activity with storage varies considerably in different preparations, but in most cases the ability to incorporate amino acids into nuclear protein is well retained for 4 to 6 hours and is almost entirely lost after 24 hours at 2°. Nuclei held at 37°C. lose their synthetic capacity more rapidly. The rate of C^{14}-alanine uptake may be cut in half after 1 hour at 37°, and be reduced to 25 per cent of the original value after 2 hours.

Synthesis vs. "Exchange".—There are several experiments now to be described which make it clear that the uptake of labelled amino acids into nuclear proteins is specific for L-amino acids and that it represents an essential aspect of protein synthesis, and is not simply a random, non-specific exchange reaction involving a few labile groups of the nucleus.

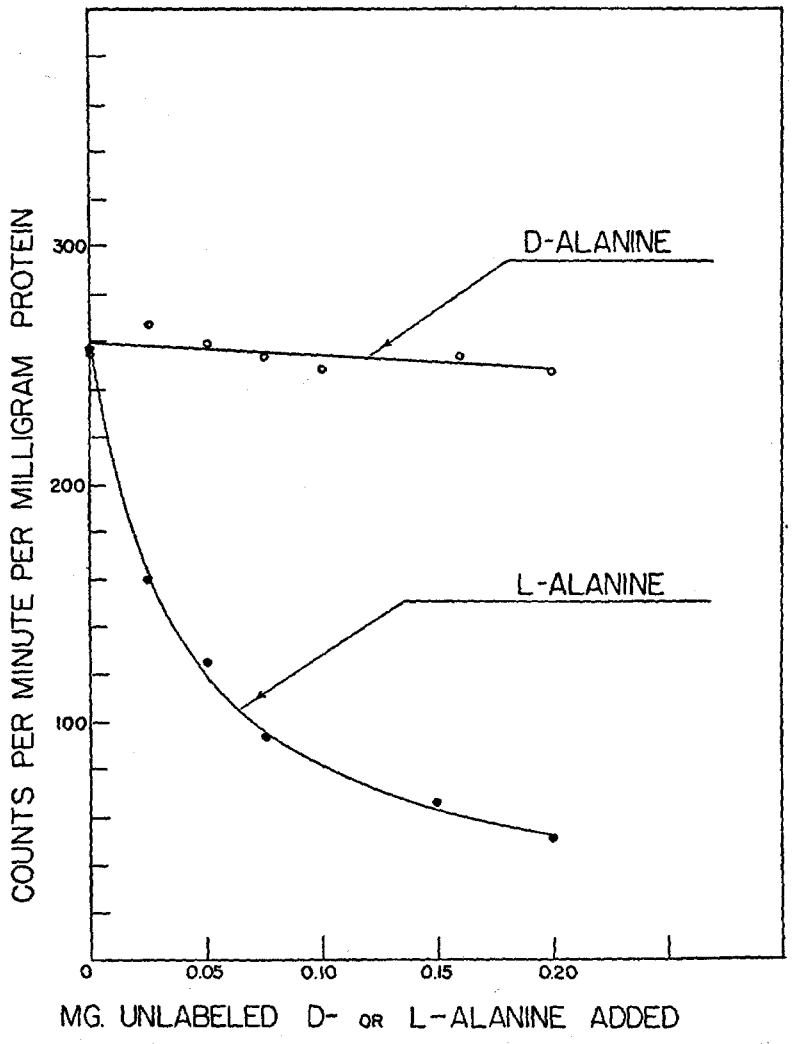

FIG. 9. The effect of unlabelled D- and L-alanines upon incorporation of DL-alanine-1-C^{14} by isolated thymus nuclei. The specific activity of the nuclear protein after 60 minutes' incubation is plotted against the amount of unlabelled D- or L-alanine added to the medium. Note that added D-alanine has no effect on C^{14}-uptake, whereas L-alanine competes with isotopic molecules for acceptance by the nucleus. It follows that only the L-isomer of the administered C^{14}-alanine is utilized for nuclear protein synthesis.

The first of these experiments is the demonstration that only the L-isomer of the administered DL-alanine-1-C^{14} is incorporated into nuclear protein. The ability of the nucleus to distinguish between D- and L- forms of the amino acid is indicated by the experiments summarized in Fig. 9. In these tests

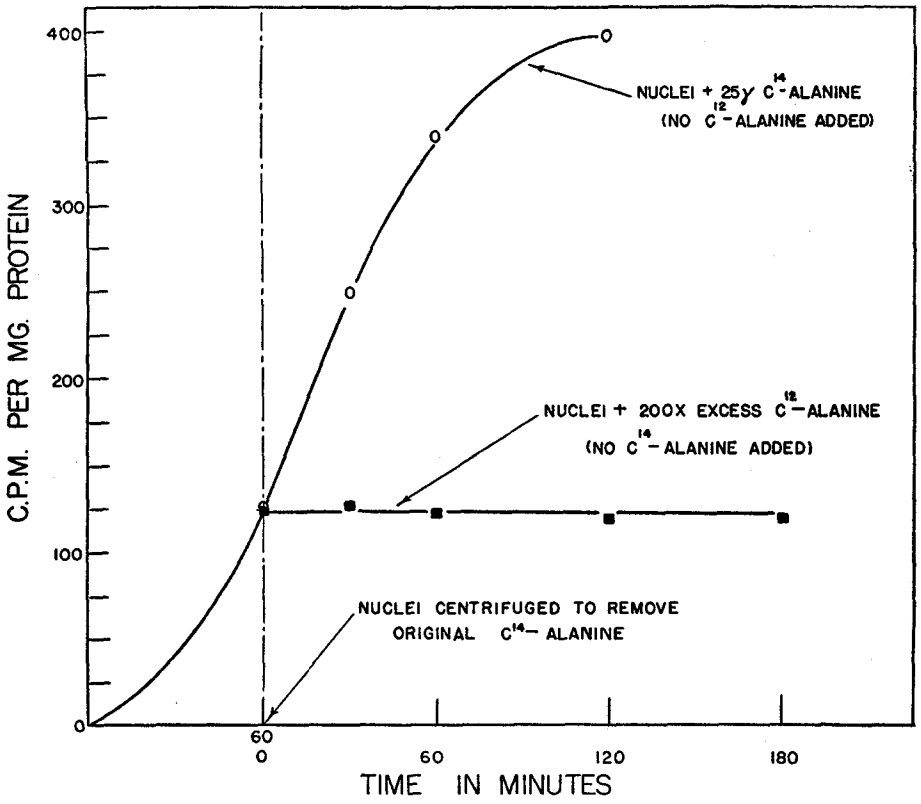

FIG. 10. The retention of incorporated alanine-1-C^{14} following addition of a large excess of unlabelled L-alanine (see text). The specific activity of the nuclear protein at different intervals after adding the unlabelled alanine is plotted against time. The upper curve shows that the nuclei in these experiments are still able to incorporate C^{14}-amino acid.

increasing amounts of unlabelled D-alanine or unlabelled L-alanine were added to the incubation medium together with the DL-alanine-1-C^{14}. If both the D- and L- forms of the C^{14}-alanine were utilized by the nucleus then both unlabelled isomers should compete with isotopic molecules for acceptance by the nucleus, and the C^{14}-activity of the nuclear protein should be correspondingly lowered. Actually, the addition of unlabelled D-alanine has no such effect; only the L-isomer competes for incorporation. It follows, therefore,

that only the L-form of the C^{14}-alanine administered is utilized for protein synthesis by the nucleus.

A second experiment was designed to test whether amino acid once incorporated is in constant exchange with other amino acid molecules in the medium. If the uptake of amino acids is indeed reversible then C^{14}-alanine once incorporated into nuclear proteins should be exchanged with unlabelled L-alanine added to the medium after incorporation has occurred. This was tested in the experiments summarized in Fig. 10. Incorporation of C^{14}-alanine was allowed to proceed for 60 minutes. At that time the nuclei were centrifuged down and the isotopic alanine was removed. The nuclei were then resuspended in the presence of a 200-fold excess of unlabelled L-alanine. Samples of the nuclei were removed at 30 minute intervals and the carbon-14 activity of the nuclear protein measured in the usual way. It is evident from the curves that the specific activity of the nuclear proteins remains essentially constant for 3 hours even in the presence of a great excess of unlabelled alanine. It follows that the C^{14}-alanine incorporated into nuclear protein is not subject to selective replacement by unlabelled alanine in the medium. The figure also shows that when additional C^{14}-alanine is added to the nuclei in such experiments, incorporation proceeds in the usual way. Thus the nuclei will continue to incorporate C^{14}-amino acid under conditions where they will not give it up. Similar experiments have been performed using S^{35}-labelled methionine and parallel results were obtained. It follows that the incorporation of isotopic amino acids into the proteins of isolated nuclei is essentially irreversible, and the possibility can be excluded that a few active centers in the nuclear protein are in constant amino acid exchange with the medium.

(It should be noted, however, that experiments of this kind do not exclude the possibility of a slow, random "turnover" involving a large number of amino acid residues, all equally subject to replacement; because if a great number of the alanine or methionine residues in the nucleus are able to exchange, and only a few of these turn over in the 3 or 4 hour experimental period, then the rate of loss of C^{14}-alanine or S^{35}-methionine once incorporated will be too small to be followed experimentally.)

It may be of value at this point to clarify our usage of the term "protein synthesis." In all experiments in which one measures the incorporation of labelled amino acids into proteins, the question naturally arises as to the relationship between the uptake observed and the synthetic process. In the strictest sense "synthesis" can be applied only in cases in which a net production of new protein molecules can be demonstrated. When such synthesis occurs in the presence of isotopically labelled amino acids the protein formed will be labelled. However, the incorporation of isotopic amino acids does not, in itself, constitute a proof that new protein molecules have been synthesized. Although in these experiments on isolated nuclei, a net synthesis of new protein has not been directly demonstrated, a number of observations make it most prob-

able that we are dealing with a very direct aspect of the synthetic mechanism: (1) The uptake of amino acids is energy-dependent. (2) The amount of amino acid incorporated into definite proteins increases with time. When the proteins of the nucleus are fractionated before counting (see below), one observes a progressive time course of amino acid uptake into such well defined protein fractions as the arginine- and lysine-rich histones. (3) The relative rates of amino acid uptake into different nuclear proteins *in vitro* are in accord with the rates observed in *in vivo* experiments. (4) Isotopic amino acid molecules once incorporated are not rapidly lost by exchange with unlabelled molecules in the medium.

From the amount of isotopic alanine incorporated an estimate can be made of the amount of new protein which may be formed. In a typical experiment 30 mg. of nuclei (10^9 nuclei) incorporate 0.6 μg. (2.4 per cent) of the administered C^{14}-alanine in 1 hour. If the average nuclear protein contains about 7.6 per cent alanine (15), this uptake corresponds to the formation of about 8 μg. of protein per hour, an amount too small to be demonstrated directly by methods now available to us. Nevertheless, the extent of the synthesis can be better appreciated from the calculation that each nucleus synthesizes 22 molecules of protein of average molecular weight 50,000 every second.

Attempts have been made to increase the extent of incorporation of isotopic alanine and glycine by the addition of a supplement of mixed L-amino acids to the nuclear suspension. No stimulation was observed, which leads to the conclusion that, if the uptake of labelled amino acids represents a net synthesis of protein, the other amino acids required must be present in the nuclei. That this is indeed the case for thymus nuclei isolated by the Behrens' procedure has been recently shown by Kay (16).

The Role of Deoxyribonucleic Acid in Protein Synthesis.—The notion originally suggested by Brachet and by Caspersson that ribonucleic acids play a role in protein synthesis is now widely accepted as demonstrated. The most direct evidence in its favor stems from the work of Gale and Folkes on amino acid uptake by bacterial cell residues (17), and from work in this laboratory on amino acid incorporation by a ribonucleoprotein complex of the liver (18). In both cases ribonuclease acts to suppress amino acid incorporation.

The isolated cell nucleus affords a unique opportunity to test the role of the deoxyribonucleic acids in the process of protein synthesis, and the results have a special interest because they ultimately bear on the mode of action of the gene, and on the chemical relationships between the nucleus and the cytoplasm.

A number of experiments which relate deoxyribonucleic acid to the process of protein synthesis in the nucleus will now be described. Several of these experiments deal with the effect of deoxyribonuclease on the uptake of labelled amino acids. When isolated nuclei are treated with crystalline pancreatic

deoxyribonuclease before adding isotopic amino acids, the incorporation of the latter is markedly impaired. The degree of impairment becomes more serious as more and more of the deoxyribonucleic acid is removed. Experiments showing the relationship between loss of DNA and inhibition of alanine-1-C^{14} and lysine-2-C^{14} uptakes are summarized in Fig. 11. At the outset there

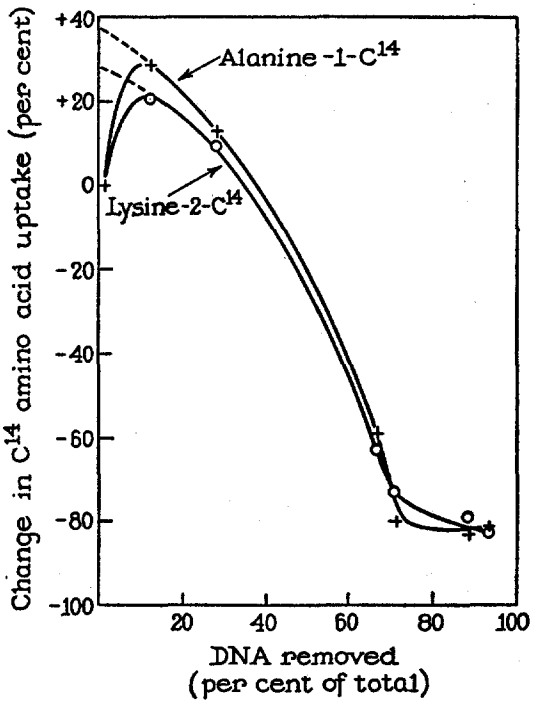

FIG. 11. The effect of removing DNA from thymus nuclei upon the subsequent incorporation of alanine-1-C^{14} and lysine-2-C^{14}. Nuclei were pretreated with DNAase to remove increasing amounts of their DNA. They were subsequently incubated for 60 minutes in the presence of the isotopic amino acid. The decrease in specific activity of the nuclear protein in treated nuclei, relative to that observed in untreated, control nuclei, is plotted against the per cent removal of the DNA.

is roughly a 1:1 correspondence between the per cent DNA removed and the per cent inhibition of the uptake. The inhibition becomes more marked when 30 to 80 per cent of the DNA is removed. Beyond that point the loss of DNA has little effect on the uptake, which remains constant at about 15 to 20 per cent of that observed in control experiments. This sensitivity of the nucleus to treatment with deoxyribonuclease is further evidence for the absence of appreciable whole cell contamination, because intact cells are not

sensitive to treatment with this enzyme. Thymus tissue slices or minces, for example, show only a very slight inhibition of alanine-1-C^{14} uptake following treatment with deoxyribonuclease, and the inhibition observed (about 15 per cent) could easily be attributed to enzyme attack of cells which had been damaged mechanically.

The foregoing experiments suggest that deoxyribonucleic acid plays a vital role in nuclear protein synthesis. Additional support for this conclusion arises

TABLE V

Effect of Supplementary Calf Thymus DNA in Restoring C^{14}-Alanine Uptake in DNAase-treated Nuclei

Supplement tested	Specific activity of nuclear protein			Activity lost	Activity regained	Recovery of lost activity
	Control nuclei	DNAase-treated nuclei	Treated nuclei + supplement			
	C.P.M./mg.	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*per cent*
1. Thymus DNA	99	15	55	84	40	48
2. " "	135	21	93	114	72	64
3. " "	99	33	101	66	68	103
" " alkali-denatured	99	33	104	66	71	108
4. Thymus DNA	40	10	29	30	19	63
" " apurinic acid	40	10	17.4	30	7.4	25
5. Thymus DNA	38	7.5	28	30.5	20.5	67
" " core after DNAase digestion	38	7.5	24.5	30.5	17	56
Thymus DNA dialyzable products of DNAase digestion	38	7.5	29	30.5	21.5	70

from experiments which test whether the synthetic activity of nuclei treated with deoxyribonuclease can be restored by the addition of supplementary DNA. In these experiments the nuclei were treated with deoxyribonuclease until 70 per cent or more of the DNA had been released into the medium. The nuclei were then centrifuged down, and the medium containing the DNA hydrolysis products was removed. The nuclei were then resuspended in the presence of a calf thymus DNA preparation: isotopic amino acid was supplied and incorporation allowed to proceed for 60 minutes. The specific activity of the protein in nuclei which had received the deoxyribonucleic acid supplement was then compared with the protein activities in control nuclei which had been treated with the enzyme in the same way but which had not received

any additional deoxyribonucleic acid. The data in Table V make it clear that added calf thymus DNA does, in fact, restore much, and at times, nearly all the original activity of the nucleus. Other experiments were performed to measure the amount of added nucleic acid taken up by DNAase-treated nuclei (Table VI). The results show that under the conditions used in all the "restoration" experiments, 30 to 40 per cent of a 5 mg. DNA supplement enters the nuclei.

It is a matter of some interest that when thymus nuclei in sucrose solution are treated with DNAase, they lose 90 per cent of their DNA and yet no more

TABLE VI

Combination of Nucleic Acids with DNAase-Treated Nuclei

Conditions of experiment	Amount of DNA in nuclei	Amount of added DNA combined	Per cent of added DNA combined
	mg.	*mg.*	
Control nuclei	11.2	—	—
Nuclei treated with DNAase	2.6	—	—
DNAase-treated nuclei + 5 mg. thymus DNA	4.13	1.63	33
" " + 10 " " "	4.6	2.0	20
DNAase-treated nuclei + 5 mg. alkali-denatured DNA	4.5	1.9	38
" " + 10 " " "	5.0	2.4	24
" " + 5 mg. yeast RNA	4.3	1.7	34
" " + 10 " " "	5.6	3.0	30

than 15 per cent of the histone is released, although histones are water-soluble and known to be attached to the DNA in the nucleus. This contrasts strongly with the properties of the same nuclei in a saline medium, for when DNA is removed and sucrose is not present, half of the histone is released from the nuclei.

This ability of the thymus nucleus in sucrose to retain its histones despite the removal of DNA may account for the ease with which added DNA is taken up by DNAase-treated nuclei, and it suggests that the proteins of the chromosome are still arranged in a specific and functional configuration.

Pretreatment of isolated thymus nuclei with deoxyribonuclease affects the uptake of different amino acids to different extents (Table VII). Methionine-S^{35} incorporation, for example, was reduced by only 42 per cent under conditions where alanine-1-C^{14} uptake was inhibited 75 per cent. But, despite this

difference, the addition of a DNA supplement seems to restore the lost activity equally in both cases.

Several experiments were performed to test whether chemically or physically modified DNA could restore the uptake in DNAase-treated nuclei. It was found that DNA denatured by treatment with alkali (pH 12.2 for 16 hours (19)) was just as effective as the original DNA preparation. Apurinic acid, while not as effective as intact DNA, does produce an appreciable stimulation of uptake in the DNAase-treated nuclei. The non-dialyzable DNA "core" which remains after exhaustive digestion of thymus DNA with deoxyribo-

TABLE VII

Effect of DNAase Treatment on Alanine-1-C^{14} and Methionine-S^{35} Incorporation by Isolated Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	DNAase / control	Activity lost	Activity regained	Recovery of last activity
		C.P.M./mg.	*per cent*	*C.P.M./mg.*	*C.P.M./mg.*	*per cent*
Control nuclei	DL-Alanine-1-C^{14}	40	—	—	—	—
Nuclei pretreated with DNAase	"	10	25	30	—	—
DNAase-treated nuclei + thymus DNA	"	29	—	30	19	63
Control nuclei	L-Methionine-S^{35}	141	—	—	—	—
Nuclei pretreated with DNAase	"	82	58	59	—	—
DNAase-treated nuclei + thymus DNA	"	119	—	59	37	63

nuclease (20), and the dialyzable split products of the digestion are both able to replace the "whole" DNA in this system (Table V).

Since even degraded thymus DNA can function in restoring amino acid uptake, it was also a matter of considerable interest to test whether nucleic acids other than calf thymus deoxyribonucleic acid can restore the synthetic capacity of DNAase-treated nuclei. We first compared calf thymus DNA with the DNA of wheat germ and found the latter to be far less effective than the former. However, when the study was extended to include DNAs from other sources, it was found that all the other nucleic acids tested were quite comparable to thymus DNA in their ability to restore C^{14}-amino acid incorporation. A number of such experiments are summarized in Table VIII. Even sea urchin sperm DNA was as effective as the DNA of the thymus.

The next step was to test the effect of ribonucleic acids on amino acid uptake by DNAase-treated nuclei. Two preparations were tested, one from calf liver,

and the other from yeast. Both were just as effective as the DNA of the thymus (Table IX). Since RNA could replace DNA in this system an attempt was made to substitute a mixture of mononucleotides for the RNA. This mixture was made up of the nucleoside 2′ or 3′ phosphates in the same proportions in which they occur in yeast RNA. These did not restore uptake. By the same token, RNA hydrolyzed in dilute alkali to yield the mononucleotides could not restore alanine incorporation in DNAase-treated nuclei.

Other compounds tested without effect included AMP, ADP, and ATP, and mixtures of the purine and pyrimidine bases. Several dinucleotides prepared from yeast RNA were made available to us through the generosity of Dr. R. B. Merrifield of The Rockefeller Institute. These included adenylic-adenylic dinucleotide, adenylic-guanylic dinucleotide, adenylic-uridylic dinucleotide, and guanylic-cytidylic dinucleotide. None of these compounds could restore uptake in DNAase-treated nuclei.

TABLE VIII

Effect of Different DNAs in Restoring C^{14}-Alanine Uptake in DNAase-treated Nuclei

Supplement tested	Specific activity of nuclear protein			Activity lost	Activity regained	Recovery of lost activity
	Control nuclei	DNAase-treated nuclei	Treated nuclei + supplement			
	C.P.M./mg.	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*per cent*
1. Thymus DNA	145	69	101	76	32	42
Wheat germ DNA	145	69	86	76	17	22
2. Thymus DNA	91	25	70	66	45	68
Calf kidney DNA	91	25	70	66	45	68
Chicken erythrocyte DNA	91	25	60	66	35	53
Paracentrotus sperm DNA	91	25	72	66	47	71
3. Thymus DNA	135	21	93	114	72	63
Trout sperm DNA	135	21	89	114	68	60

Although fragments as small as dinucleotides do not seem to be active in our system (with the reservation that only a few of the possible dinucleotides were tested), it was found that the dialyzable split products of RNA digestion by RNAase were partially effective; and it has already been pointed out that the dialyzable split products of DNA digestion by DNAase were quite effective. Both these findings suggest that relatively simple, low molecular weight compounds, perhaps of the order of trinucleotides, are able to substitute for DNA in mediating or facilitating amino acid incorporation.

Further fractionation studies are now in progress in an attempt to further characterize and isolate the active dialyzable material.

That intact nucleic acids have a high degree of specificity in biological systems is evident both from the role of DNA in bacterial transformation (21), and of RNA in tobacco mosaic virus infection (22–24). In the amino

TABLE IX

Effect of Ribonucleic Acids and Ribonucleotides on C^{14}-Alanine Uptake by DNAase-treated Nuclei

Supplement tested	Specific activity of nuclear protein			Activity lost	Activity regained	Recovery of lost activity
	Control nuclei	DNAase-treated nuclei	Treated nuclei + supplement			
	c.p.m./mg.	*c.p.m./mg.*	*c.p.m./mg.*	*c.p.m./mg.*	*c.p.m./mg.*	*per cent*
1. Calf liver RNA	92	54	80	38	26	68
" thymus DNA	92	54	81	38	27	71
2. Thymus DNA	68	23	54	45	31	69
Yeast RNA	68	23	58	45	35	78
" " hydrolyzed in 0.3 N NaOH	68	23	23	45	0	0
Yeast RNA dialyzable after RNAase digestion	68	23	36	45	13	29
A, G, U, C, mononucleotide mixture	68	23	23	45	0	0
3. Thymus DNA	83	14	46	69	32	46
Yeast RNA	83	14	45	69	31	45
Adenylic-adenylic dinucleotide	83	14	18	69	4	6
Adenylic-guanylic dinucleotide	83	14	18	69	4	6
Adenylic-uridylic dinucleotide	83	14	18	69	4	6
Guanylic-cytidylic dinucleotide	83	14	18	69	4	6

acid incorporation system which we have described there is, as yet, no inkling of such specificity. On the contrary, the findings in nuclei deprived of their original DNA suggest a rather generalized ability of polynucleotide fragments to facilitate amino acid incorporation. In the intact nucleus this ability is very probably linked with factors of specificity in the intact DNA. Yet the ability of other DNAs, DNA fragments, and RNA to substitute for thymus DNA in these experiments introduces some questions of considerable interest. Does specificity reside in the chromosomal protein as well as in the DNA? Evidence has already been presented to show that even the smaller proteins of the

chromosome are retained by DNAase-treated nuclei. If the proteins originally associated with DNA retain their specific configurations, then added polynucleotide may be taken up only to conform with the original spatial arrangement of the native DNA. Moreover, added polynucleotides might serve to stabilize synthetic "machinery" which would be extremely labile once the native DNA had been removed. If this is the case, then the proteins synthesized by "restored" DNAase-treated nuclei might be the same, regardless

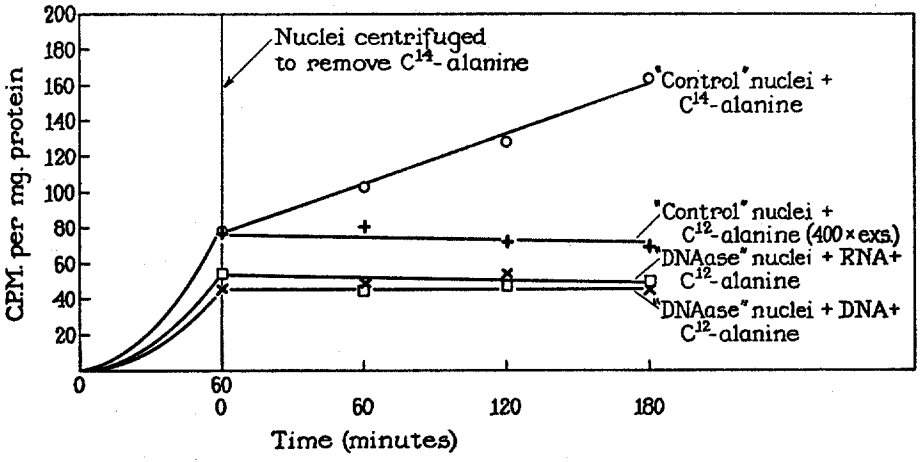

FIG. 12. The retention of the alanine-1-C^{14} incorporated into the proteins of DNAase-treated nuclei which were restored by adding thymus DNA or yeast RNA. (See text.) The nuclei were treated with DNAase, then given a nucleic acid supplement and incubated in the presence of C^{14}-alanine. They were then centrifuged down and reincubated in the presence of a great excess of unlabelled L-alanine. The specific activity of the nuclear protein at different intervals after adding the unlabelled alanine is plotted against time. The upper curve shows that the nuclei in these experiments are still able to incorporate C^{14}-amino acid.

of the source of added nucleic acid. On the other hand, if specificity resides only in the nucleic acid, then the possibility exists that added nucleic acids lead to the synthesis by the nucleus of different proteins. This would be analogous to bacteriophage DNA dominating and modifying the metabolism of the cell which it infects. This possibility that different proteins are made in the presence of different nucleic acids is being investigated. An alternate possibility, of course, is that the nucleus uses added polynucleotides for the synthesis of its own, characteristic DNA (but cannot use nucleoside 2′ or 3′ phosphates). The ability of isolated nuclei to synthesize nucleic acids from small precursors is demonstrated below.

The ability of RNA to restore alanine uptake in nuclei deprived of their

DNA naturally raises the question of the role of ribonucleic acid in nuclear metabolism. Isolated thymus nuclei contain only a small amount of ribonucleic acid. (Analysis of the RNAase digest of acid-washed nuclei, using the orcinol procedure, shows an RNA content equal to 1.4 to 2.2 per cent of the lipid-free dry weight. The more specific *p*-bromophenylhydrazine method recently introduced by Webb (37) shows an RNA content of 1.4 per cent.) At-

TABLE X

Effect of Methyl Green, Protamine, and a Histone upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	Change in C^{14} uptake
		C.P.M./mg.	*per cent*
1. Control nuclei	DL-Alanine-1-C^{14}	154	—
Nuclei + 0.125 mg. methyl green	"	167	+8
" + 0.25 " " "	"	162	+5
" + 0.50 " " "	"	116	−25
2. Control nuclei	Glycine-1-C^{14}	205	—
Nuclei + 0.25 mg. methyl green	"	251	+22
3. Control nuclei	"	89	—
Nuclei + 0.50 mg. methyl green	"	64	−28
4. Control nuclei	DL-Alanine-1-C^{14}	154	—
Nuclei + 0.375 mg. protamine	"	168	+9
" + 0.75 " "	"	159	+3
" + 1.50 " "	"	164	+6
5. Control nuclei	Glycine-1-C^{14}	79	—
Nuclei + 1.5 mg. protamine	"	70	−11
" + " " histone (lysine-rich)	"	76	−4

tempts to influence nuclear protein synthesis by removing this small amount of RNA have not so far been successful. Treatment of the nuclei with ribonuclease before adding C^{14}-amino acids has no effect on the uptake of the latter. However, this result does not permit the conclusion that ribonucleic acid has no part in nuclear protein synthesis, because in the experiments mentioned only 54 per cent of the nuclear RNA could be released by the action of the enzyme. The refractory nature of RNA-binding in the thymus nucleus is also evidenced by the fact that the DNA can be removed without appreciably influencing the RNA content. There is, however, another line of attack, directed against nuclear RNA synthesis, and evidence is presented below which suggests that the RNA of the nucleus has an important role in initiating the synthesis of some nuclear proteins.

It was previously noted that the uptake of isotopic amino acids into the proteins of thymus nuclei is not a rapidly reversible, exchange type reaction. This conclusion was obtained from experiments which showed that labelled amino acids once incorporated into nuclear proteins are not readily replaced by non-labelled amino acids in the medium. Similar experiments were performed on DNAase-treated nuclei which were supplemented with either thymus DNA or yeast RNA. These tests are summarized in Fig. 12. In both cases the uptake was apparently irreversible: the nuclei did not lose isotopic amino acid once it was incorporated. The failure to exchange cannot be the result of a loss of activity because such nuclei given C^{14}-alanine show an extensive C^{14} uptake into their proteins.

Since, in the intact nucleus, deoxyribonucleic acid is required for protein synthesis the question naturally arises as to how the DNA participates in amino acid incorporation. Several experiments were performed to test whether the action of deoxyribonucleic acid requires the active participation of its free phosphate groups. The results of these experiments are summarized in Table X. The procedure used was to add varying amounts of basic compounds which are known to combine with the free phosphoric acid groups of deoxyribonucleic acid. The compounds selected included the basic dye, methyl green, and two basic proteins, protamine, and a lysine-rich histone prepared from calf thymus. It is clear that the addition of small amounts of methyl green, protamine, or histone to the nuclei before adding the isotopic amino acid has little effect on the uptake. Larger amounts lead to a partial impairment of carbon-14 incorporation; but the impairment may reflect osmotic or other damage to the system, since large amounts of added serum albumin also decrease the uptake. The results suggest that some of the free phosphate groups of the DNA can be blocked without affecting the uptake of labelled amino acids.

Which Nuclear Proteins Become Labelled?—The answer to this question depends at the outset upon the existence of a dependable and meaningful scheme of fractionating the proteins of the nucleus. This is a field of endeavor which is now in its earliest stages of development, though it promises to become a major topic of biochemical investigation.

We have developed a provisional fractionation procedure which is applicable to the proteins of thymus sucrose nuclei, and which permits the separation of several classes of proteins of different properties and considerable biological interest. In its essentials the procedure is as follows: (See flow-sheet in Fig. 13.)

1. The nuclei are incubated in buffered sucrose solution in the presence (in these experiments) of C^{14}-alanine. After uptake has occurred the nuclei are centrifuged down. The protein released into the medium is precipitated with trichloroacetic acid (TCA). The amount of this protein in the medium increases with the time of incubation, but it has a very low specific activity

relative to the other proteins of the nucleus. The uptake into this fraction does not seem to be dependent upon DNA since pretreatment of the nuclei with DNAase does not affect it; nor does RNAase have any effect.

2. The nuclei are next extracted with 0.1 M potassium phosphate buffer at pH 7.1. The extract contains some RNA and a small amount of protein, the yield of which seems to diminish as the incubation is prolonged. This protein

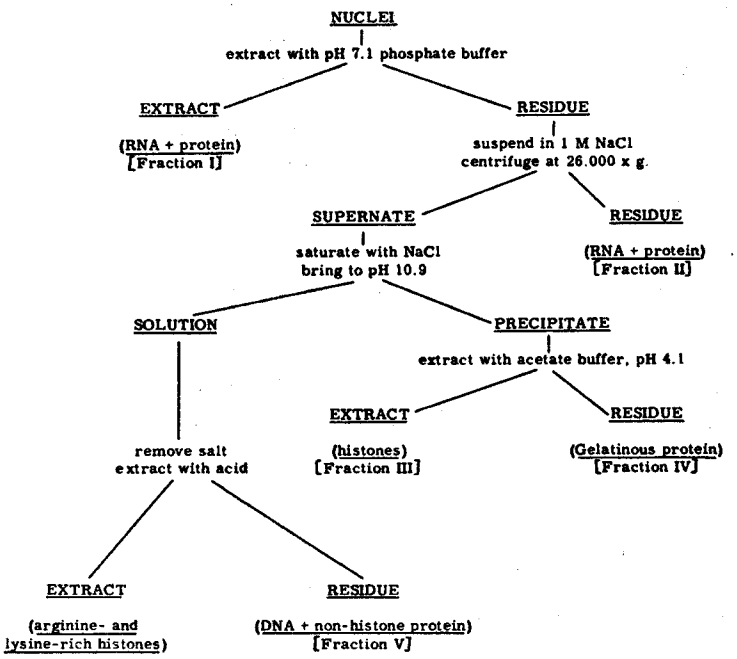

FIG. 13. Flow-sheet for the fractionation of the proteins of isolated thymus nuclei (see text).

[fraction I] has considerable interest because at times it is the most actively labelled fraction of the nucleus. Moreover, the uptake of isotope into this fraction is almost completely abolished by pretreating the nuclei with DNAase. It is not affected by pretreatment with RNAase.

3. The nuclei are then suspended in 1 M sodium chloride solution, in which they swell and largely dissolve. The opalescent solution is centrifuged at 26,000 $\times$ g to sediment the undissolved material. This residue [fraction II] comprises about 10 per cent of the nuclear mass and contains one-third of the nuclear ribonucleic acid. There is evidence to suggest that this fraction contains the nucleoli (25).

4. The supernatant after high speed centrifugation contains the bulk of the protein of the nucleus, and all the deoxyribonucleic acid. It can be frac-

tionated to yield different histones, a gelatinous protein, and an unfractionated residue. The protein in the latter is firmly attached to the DNA. (See reference (2) and experimental section.)

The distribution of C^{14} activity in these different protein fractions is a

TABLE XI

Alanine-1-C^{14} Incorporation into Different Protein Fractions of Thymus Nuclei

Protein fraction	Incubation time	Specific activity
	min.	*c.p.m./mg.*
1. Total nuclear protein	90	107
Fraction I (protein-soluble in pH 7.1 buffer)	"	179
" II (residue after extraction in 1 M NaCl)	"	117
" III (histones)	"	36
" IV (gelatinous protein)	"	157
" V (non-histone protein associated with DNA)	"	243
2. Protein released to medium during incubation	30	5
	60	8
	90	16
Fraction I	30	10
	60	66
	90	105
Arginine-rich histones	30	2.3
	60	4.5
	90	6.7
Lysine-rich histones	30	—
	60	5.6
	90	6.9
3. Control nuclei		
Protein released to medium during incubation	90	41
Fraction I	"	323
Other fractions combined	"	64
DNAase-treated nuclei		
Protein released to medium during incubation	90	47
Fraction I	"	25
Other fractions combined	"	5

matter of some interest (Table XI). (It should be mentioned that the total alanine contents of the protein fractions are very similar, so that differences in carbon-14 concentration reflect differences in the metabolic activity of the various proteins.) It was found that the protein most closely associated with the DNA is more active than all other proteins of the nucleus, with the occasional exception of the small fraction extractable in pH 7.1 buffer. This result lends further support to the conclusion drawn from deoxyribonuclease experiments that the DNA plays a role in protein synthesis. A second point

of interest is the fact that the level of isotope incorporation into the histone proteins is relatively low. This result agrees with earlier *in vivo* experiments in which it was noted that the incorporation of N^{15}-glycine into the histones of mouse liver, pancreas, and kidney is much lower than the uptake into the residual proteins of the chromosome (26).

A final point deals with the effect of pH upon amino acid incorporation into the different protein fractions of isolated nuclei. When nuclei are incubated at pH 6.7, the uptake of C^{14}-alanine into the DNA-attached protein is only about half of that observed following incubation at pH 7.3. In contrast, the uptake into the proteins of the "pH 7.1 extract" is slightly higher when the nuclei are incubated at pH 6.7.

Nucleic Acid Synthesis.—The question now arises as to whether the isolated thymus nucleus can synthesize nucleic acid by itself. This was tested by incubating the nuclei in the presence of C^{14}-labelled glycine, a substance known to be a precursor of the purine ring in nucleic acid biosynthesis. Table XII summarizes some preliminary experiments in which incorporation was demonstrated in three ways. In the first, the total nucleic acid was extracted with dilute KOH and prepared by zone electrophoresis. It may contain a trace of adsorbed protein. In the other experiments the nucleotides and the purines were separated on ion exchange resins. In all cases, a detectable amount of isotope appeared in the nucleic acid.

TABLE XII

Incorporation of Glycine-1-C^{14} into the Proteins and Nucleic Acids of Isolated Calf Thymus Nuclei

Nuclear component	Specific activity
	c.p.m./mg.
Total nuclear protein	117
Nucleic acid prepared by zone electrophoresis	19
Nucleotides prepared on dowex 1	11
Purines prepared on dowex 50	57

A further study of nucleic acid synthesis in isolated nuclei was carried out using orotic acid–6-C^{14} as precursor of the nucleic acid pyrimidines. In these experiments the uptake of radiocarbon into the *ribonucleic* acids of the nucleus was followed by incubating nuclei at 37° in the usual buffered sucrose medium in the presence of orotic acid–6-C^{14}. After washing to remove acid-soluble nucleotides, the nuclear RNA was prepared by treating the nuclei with ribonuclease: the released RNA fragments were adsorbed on charcoal for counting. We also found that some nucleic acid was readily extracted from acid-washed nuclei when they were placed in 0.2 M phosphate buffer at pH 7. This material was adsorbed and counted separately. The results of several such experiments are summarized in Table XIII. Separate columns

list the specific activity of RNA selectively released by RNAase, the nucleic acid extracted by the buffer, and the combined fractions. It is clear that a rapid and considerable uptake of orotic acid takes place, and that there is a well defined time course of C^{14} incorporation into nuclear RNA.

TABLE XIII

Orotic Acid-6-C^{14} Incorporation into Nuclear Ribonucleic Acids

Conditions of experiment	Incubation time	Specific activity of		
		RNA released by RNAase	NA soluble in buffer	Combined NA fractions
	min.	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*
1. Control nuclei*	30	85	202	147
	60	142	306	220
	90	385	420	402
	120	443	472	457
Nuclei + 0.1 mg. DRB at t = 0 min.	30	0	136	62
	60	0	191	87
	90	93	234	161
	120	180	237	206
Nuclei + 0.1 mg. DRB at t = 30 min.	30	89	202	144
	60	93	—	152
	90	93	287	176
	120	54	349	179
2. Control nuclei‡	30	290	1270	567
	60	625	1640	938
	90	690	1750	1030
Nuclei + 0.1 mg. DRB at t = 0 min.	30	91	715	253
	60	100	925	288
	90	225	833	427
Nuclei + 0.1 mg. DRB at t = 30 min.	30	364	1270	641
	60	397	1105	617
	90	339	873	500

* Each flask (containing 30 mg. of nuclei in 2.0 ml. of incubation medium) received 0.075 mg. orotic acid-6-C^{14}; specific activity 1.0 mc./mM.

‡ Each flask received 0.20 mg. orotic acid-6-C^{14}.

The synthesis of ribonucleic acid by the nucleus seems to require the presence of deoxyribonucleic acid. In preliminary experiments it was found that pretreatment of the nuclei with DNAase greatly reduced subsequent orotic acid incorporation into the RNA. For example, in nuclei which had lost 68 per cent of their DNA, the ribonucleic acid had a specific activity of only 21 C.P.M. per mg. after 1 hour's incubation. The corresponding figure for un-

treated controls was 186 C.P.M. per mg. of RNA. The effect of a DNA supplement on nucleic acid synthesis by DNAase-treated nuclei has not yet been tested. (In a recent communication, Friedkin and Wood report the incorporation of C^{14}-thymidine into the DNA of isolated rabbit thymus nuclei. In their experiments, too, the presence of DNAase lowers the incorporation (36).)

It was previously pointed out that 5,6-dichloro-β-D-ribofuranosyl-benzimidazole (DRB) is an inhibitor of protein synthesis in isolated thymus nuclei, and that one of the most striking properties of this inhibitor is the time limit of its effectiveness. DRB is an effective inhibitor of nuclear protein synthesis only if it is added at the outset of the incubation. If alanine-C^{14} uptake is allowed to proceed before adding the DRB, little or no subsequent inhibition is observed. These results suggest that in the first 30 minutes some activation or "priming" process takes place within the nucleus which permits protein synthesis. Once the activation has occurred, DRB cannot inhibit further amino acid uptake.

Now what is the nature of the "DRB-sensitive" activation process? The structure of DRB suggests that it may function as a purine nucleoside antagonist and interfere with the synthesis of nucleic acid. To test this hypothesis we studied the effect of DRB on RNA synthesis by isolated nuclei. The results are included in Table XIII. In control nuclei one observes a straightforward time course of orotic acid-C^{14} incorporation into nuclear RNA. But when DRB is added to the medium at the outset of the experiment, C^{14} uptake is greatly reduced. The addition of DRB 30 minutes after the beginning of the experiment also effectively inhibits further RNA synthesis. Thus DRB seems to block the synthesis of ribonucleic acid within the nucleus no matter when it is added to the incubation medium. On the other hand, DRB inhibits nuclear protein synthesis only when it is present from the beginning.

These results suggest that the activation or priming of the nucleus so that it actively incorporates amino acids into its proteins requires a preliminary synthesis of ribonucleic acid. Once this nucleic acid has been laid down, protein synthesis begins. Subsequent interference with RNA synthesis does not appreciably affect the level of amino acid uptake (in the time interval studied in these experiments). The question now arises as to whether the "activation" of the nucleus involves the synthesis of an autonomous system, which then takes over protein synthesis independently of the DNA. This was tested in experiments in which the DNA was not removed until activation had occurred. It was found that removal of the DNA after a preliminary 30 minute incubation stopped protein synthesis just as effectively as it always does in unincubated samples. It follows that the requirement for DNA in nuclear protein synthesis remains even after activation has occurred. Our studies have shown that the DNA of the nucleus is essential for both RNA and pro-

tein synthesis. It remains to be seen whether DNA *synthesis* is required for any of these essential biosynthetic reactions.

EXPERIMENTAL

Preparation of Nuclei.—The procedure used is a modification of that described by Schneider and Petermann (29). All operations were carried out at 2°C. 50 grams of fresh calf thymus tissue was finely minced with scissors and placed in a blendor vessel together with 50 ml. of 0.5 M sucrose solution and 400 ml. of 0.25 M sucrose–0.0033 M $CaCl_2$. (This calcium concentration is higher than that previously described (1) because larger amounts of Ca^{++} ion prevent the gel formation occasionally obtained in the earlier procedure.) The tissue was gently homogenized by running the blendor at 1000 R.P.M. for 4 minutes. The resulting homogenate was filtered through a double layer of gauze (Johnson and Johnson type I) and then through a single thickness of double napped flannelette. The filtrate was centrifuged at 2400 R.P.M. (1000 × *g*) for 7 minutes and the supernate was discarded. The sediment was resuspended in 100 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$ solution and the suspension was again passed through flannelette. The filtrate was centrifuged at 1000 × g for 7 minutes. The sedimented nuclei were again washed in the centrifuge using 100 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$. Further washings were rarely necessary, since the supernate by this time was usually water-clear. The nuclei were finally suspended in 40 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$, a dilution which usually provided 30 to 50 mg. of nuclei (dry weight) per ml. of suspension.

Incubation Procedure.—Aliquots of the nuclear suspension were incubated aerobically at 37° in the presence of isotopically labelled amino acid, buffer, and added metabolites, as follows: each incubation vessel contained (1) 1.0 ml. of nuclear suspension, (2) 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer, (3) 0.4 ml. of 0.1 M glucose solution containing 3.75 mg. NaCl per ml., and (4) 0.1 ml. of water containing 0.05 mg. of radioactive amino acid. The amino acids used were DL-alanine-1-C^{14} (specific activity 1.4 mc./mM), glycine-1-C^{14} (specific activity 1.35 mc./mM), DL-lysine-2-C^{14} (specific activity 0.33 mc./mM), and L-methionine-S^{35} (specific activity 4.4 mc./mM). Most of the experiments described were carried out at pH 6.7 or pH 7.3. Uptakes are higher at the higher pH but under these conditions nuclei formed a heavy gel which was difficult to centrifuge and which precluded a clean separation of nuclei from suspending medium. When such separations were necessary, as in the DNAase experiments and tests for amino acid exchange, the pH was held at 6.7 and occasionally at 6.3. The flasks were immersed in a 37° water bath and shaken at 140 cycles per minute for the required period, usually 1 hour. In experiments in which the proteins of the nucleus were to be subsequently fractionated, the uptake was halted by the addition of 0.2 ml. of 0.1 M NaCN solution. In all other cases the reaction was stopped by the addition of an equal volume (2.0 ml.) of 20 per cent trichloroacetic acid.

In testing the effects of various inhibitors (Tables I and III) these substances were dissolved in the phosphate-sucrose buffer at four times the final concentrations listed in the table.

In comparing glucose, fructose, and α-ketoglutarate (Table II) these substances

were prepared as 0.1 M solutions containing the required amount of sodium: 0.4 ml. of each solution was used, together with 1.0 ml. of nuclear suspension, 0.5 ml. of buffer, and 0.1 ml. of the amino acid solution.

In testing the effects of the substituted benzimidazoles (Table IV and Fig. 5), the compounds were dissolved in 0.25 M sucrose–0.0030 M $CaCl_2$. One-tenth ml. of solution containing the indicated amounts of inhibitor was added to the incubation mixture at 0, 30, or 60 minutes. Controls received 0.1 ml. of sucrose–$CaCl_2$. The time course of incorporation in control nuclei and in nuclei receiving the inhibitor was followed by halting the uptake of different flasks at 30 minute intervals.

In studying the effect of varying sodium concentration upon amino acid uptake (Fig. 6), the incubation mixture contained 1.0 ml. of nuclear suspension, 0.5 ml. of 0.03 M potassium phosphate–sucrose buffer at pH 7.3, 0.4 ml. of 0.1 M glucose containing varied amounts of sodium chloride, and 0.1 ml. of glycine-1-C^{14} solution containing 1.0 mg. = 0.018 mc./ml. Uptake was allowed to proceed for 1 hour.

The change in amino acid incorporation with changing sodium/potassium ratios (Fig. 7) was measured in incubation mixtures containing 1.0 ml. of nuclear suspension, 0.5 ml. of 0.03 M potassium phosphate–0.175 M sucrose buffer at pH 7.3, 0.4 ml. of 0.1 M glucose containing 0.137 mM of total chlorides, the ratio of sodium to potassium being varied as indicated in the chart, and 0.1 ml. of glycine-1-C^{14} solution containing 1.0 mg. = 0.018 mc./ml.

The effect of sucrose concentration upon alanine-C^{14} uptake (Fig. 8) was studied using nuclei which had been isolated and suspended in 0.25 M sucrose–0.0030 M $CaCl_2$. The incubation mixture contained 1.0 ml. of nuclear suspension, 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml., 0.1 ml. of DL-alanine-1-C^{14} solution containing 0.5 mg. = 0.008 mc./ml., and 0.5 ml. of 0.1 M sodium phosphate buffer (pH 6.7) containing varying amounts of sucrose (between 0 and 1.5 M) to give the final concentrations plotted in the chart. Uptake was stopped after 60 minutes.

In testing whether nuclei utilize the D-form of administered amino acids (Fig. 9) the incubation mixture comprised 1.0 ml. of nuclear suspension, 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 7.3) containing varied amounts of either unlabelled D-alanine or unlabelled L-alanine, as indicated in the chart, 0.4 ml. of 0.1 M glucose containing 6.25 mg. NaCl per ml., and 0.1 ml. of DL-alanine-1-C^{14} solution containing 0.5 mg. = 0.008 mc./ml. Incorporation was allowed to proceed for 60 minutes.

The test for exchange of amino acid once incorporated (Fig. 10) was carried out as follows: nuclei were incubated in the usual way (but at pH 6.3 to minimize gel formation) for 60 minutes in the presence of 0.05 mg. of DL-alanine-1-C^{14}. At that time the suspensions were transferred to centrifuge tubes (12 ml. capacity, with conical tip) and the nuclei were sedimented at 2600 R.P.M. for 5 minutes. The supernate was removed and the nuclei were resuspended in 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$, 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 6.3), and 0.4 ml. of 0.1 M glucose containing 7.5 mg. NaCl per ml. Control flasks received 0.1 ml. H_2O. Other flasks received 0.1 ml. of the DL-alanine-1-C^{14} solution used previously (to test whether nuclei after centrifugation and resuspension could still incorporate amino acid). The experimental flasks received 0.1 ml. of unlabelled L-alanine solution containing 5 mg. alanine/ml. This supplied more than 200 times as much unlabelled

amino acid as C^{14}-alanine remaining. The nuclei were then incubated at 37°. The reaction was followed by halting the uptake of different flasks at 30 minute intervals, and the protein was prepared as described below. Other controls using unlabelled D-alanine, showed that the amount of unlabelled amino acid added did not lead to osmotic or other damage to the system.

Similar experiments, using S^{35}-labelled methionine, were carried out by the same general procedure. These experiments were performed at pH 6.7. The conclusions are essentially the same as those derived from C^{14}-alanine experiments.

In studying the effect of removing DNA on the incorporation of alanine-1-C^{14} and lysine-2-C^{14} (Fig. 11), the following procedure was used: each flask contained 1.0 ml. of nuclear suspension and 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl and 4.19 mg. $MgCl_2 \cdot 4H_2O$ per ml. Control flasks received 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer at pH 6.7. Other flasks received this buffer containing increasing amounts of crystalline pancreatic deoxyribonuclease (Worthington). Enzyme concentrations in the buffer ranged from 0.125 to 4.0 mg. per ml. The suspensions were incubated at 37° for 30 minutes, and the nuclei were centrifuged down at 2600 R.P.M. for 5 minutes. The supernates were carefully removed and the amount of nucleic acid which they contained was measured spectrophotometrically at 260 mμ. The nuclei were then resuspended in 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$ containing 2.94 mg. Na_3 citrate$\cdot 2H_2O$ per ml., 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 6.7), 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml., and 0.1 ml. of H_2O containing 0.05 mg. of either DL-alanine-1-C^{14} or DL-lysine-2-C^{14}. Incorporation was allowed to proceed for 60 minutes, when it was halted by the addition of an equal volume of 20 per cent trichloroacetic acid (TCA).

The "restoration" of amino acid incorporation in DNAase-treated nuclei (Tables V, VII, VIII, and IX) was obtained as follows: each flask contained 1.0 ml. of nuclear suspension and 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl and 4.19 mg. $MgCl_2 \cdot 4H_2O$ per ml. Control flasks received 0.5 ml. of the phosphate-sucrose buffer at pH 6.7. Other flasks received this buffer containing 2.0 mg. deoxyribonuclease per ml. The suspensions were incubated at 37° for 30 minutes, and the nuclei centrifuged down as described above. The amount of nucleic acid released into the supernate was measured by the E_{260}. The nuclei were then resuspended in 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer, 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml., and 0.1 ml. of isotopic amino acid solution. Some of the flasks received 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$ containing 2.94 mg. Na_3 citrate$\cdot 2\ H_2O$ per ml. and nothing else. Other flasks (containing DNAase-treated or control nuclei) received 1.0 ml. of the sucrose-citrate solution containing 5 mg. of DNA (or other supplement) per ml. The flasks were then incubated at 37° for 60 minutes and the uptake was stopped by the addition of an equal volume of 20 per cent TCA. The C^{14} incorporations into the proteins of the control nuclei were compared with those observed in DNAase-treated nuclei, and in treated nuclei which had received a nucleic acid supplement. The supplements used had little or no effect on amino acid uptake by control nuclei.

A number of other controls were run in these experiments. In some cases DNA was added to DNAase-treated nuclei after the incubation with labelled amino acids. The purpose of this experiment was to see whether an actively labelled fraction exists

which might be formed by DNAase-treated nuclei and be coprecipitated only in the presence of added DNA. (Such a fraction, if it existed, would have to be soluble in 10 per cent TCA.) No evidence for such an elusive, active protein was found. DNA added after incubation with C^{14}-alanine did not lead to higher specific activities of the nuclear protein. In other experiments, nuclei were allowed to incorporate C^{14}-alanine before treatment with DNAase. They were subsequently treated with the enzyme, and an active protein fraction was searched for in the medium. These experiments showed that DNAase- treated nuclei lost none of their counts: all the activity remained in TCA-precipitable nuclear protein.

The nucleic acids used were prepared by methods previously described (30, 31). The apurinic acid was prepared by the method of Tamm, Hodes, and Chargaff (32).

The test for exchange of amino acids taken up by restored DNAase-treated nuclei (Fig. 12) was carried out by combining two of the above procedures. Nuclei were treated with DNAase, centrifuged, and resuspended in the presence of thymus DNA or yeast RNA and C^{14}-alanine, as described above. After 60 minutes' incubation, the nuclei were centrifuged down, and the supernate was discarded. The nuclei were then resuspended in 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$, 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 6.7), and 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml. Control flasks received 0.1 ml. H_2O. Other flasks received 0.1 ml. H_2O containing 0.05 mg. DL-alanine-1-C^{14} (to test whether these nuclei could still incorporate amino acid). The experimental flasks received 0.1 ml. of unlabelled L-alanine solution (10 mg./ml.). This supplied more than 400 times as much unlabelled amino acid as C^{14}-alanine remaining. The nuclei were then incubated at 37°. The reaction was followed by halting the uptake of different flasks at 30 minute intervals. The protein was prepared as described below.

In testing the effects of methyl green and basic proteins upon amino acid incorporation (Table X), these substances were dissolved in the phosphate-sucrose buffer so that 0.5 ml. contained the amounts listed in the table. The methyl green was recrystallized as the perchlorate. The lysine-rich histone was prepared by procedures previously described (15). The protamine was a preparation of the Lilly Research Laboratories. Uptake was halted after 60 minutes by the addition of an equal volume of 10 per cent TCA.

Protein Fractionation.—For measuring alanine-1-C^{14} incorporation into different protein fractions of the nucleus (Fig. 13 and Experiment 1 in Table XI) the scale of the experiments was increased by a factor of 25. To 25 ml. of nuclear suspension was added 12.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 7.3), 10 ml. of 0.1 M glucose containing 6.25 mg. NaCl per ml., and 2.5 ml. H_2O containing 1.25 mg. DL-alanine-1-C^{14}. The suspension was shaken at 37° for 90 minutes. It was then cooled in an ice bath and fractionated as follows:—

1. The nuclear gel was centrifuged down, and a small portion of the sediment taken for a measure of C^{14} uptake into the total nuclear protein.

2. All subsequent operations were carried out at 2°C. The residue was minced with scissors and extracted with 50 ml. of 0.1 M potassium phosphate buffer at pH 7.1 for 10 minutes. (In later experiments this extraction was carried out by homogenizing the nuclear gel in the blendor for 30 seconds.) The suspension was centrifuged and the clear supernate was carefully decanted. This pH 7.1 extract (fraction I) was

precipitated by adding TCA to a final concentration of 10 per cent. The proteins were prepared for counting as described below.

3. To the nuclear residue an equal volume of 2 M NaCl was added. The gel was stirred in a high-speed mixer for 3 hours. It was then centrifuged at 26,000 R.P.M. in the Spinco ultracentrifuge for 2 hours. The sediment (fraction II) was washed with 1 M NaCl and prepared for counting as described below.

4. The supernate was saturated with NaCl and brought to pH 10.9 by the careful addition of 1 N NaOH. After stirring for 20 minutes, the suspension was centrifuged to remove the precipitated histones and gelatinous protein. The supernate (A) was set aside. The sediment was washed with saturated NaCl solution at pH 10.9. It was then taken up in water (about 15 ml.) and dialyzed overnight. The suspension was centrifuged, and the supernate (which contains the histones) was set aside. The gelatinous residue was extracted with 8 ml. of 0.1 M acetate buffer at pH 4.1 and recentrifuged at 12000 R.P.M. This extraction was repeated, and both extracts were added to the original supernate containing the histones. The residue of gelatinous protein (fraction IV) was prepared for counting as described below.

5. The combined supernates were brought to pH 11 with NaOH. The precipitated histones (fraction III) were washed with ethanol and prepared for counting.

6. The proteins of supernate (A) were next fractionated by adding 2 N HCl to bring the final acid concentration to 0.2 N. The DNA-protein precipitate was centrifuged down. The residue was reextracted with 0.2 N HCl, and the precipitate (fraction V) prepared for counting as described below.

7. The supernate contains both arginine-rich and lysine-rich histones, which can be fractionated by methods previously described (15).

Preparation of Protein for Counting.—In most experiments the over-all specific activity of the total nuclear protein was determined. The procedure was to halt incorporation by the addition of an equal volume of 20 per cent TCA. The suspension was brought to a volume of 10 ml. with 10 per cent TCA and centrifuged (2600 R.P.M. for 10 minutes). The supernate was discarded. The residue was washed three times in the centrifuge with 10 ml. portions of 10 per cent TCA. The washings were discarded. The residue was taken up in 10 ml. of 10 per cent TCA, brought to 90°C. for 20 minutes, and centrifuged. The supernate, which contains the nucleic acids, was discarded. The residue was again washed with 10 per cent TCA. To remove lipides the precipitate was washed once with hot 95 per cent ethanol, twice with a warm 2:2:1 ethanol-ether-chloroform mixture, and once with ether. The protein was then air-dried at room temperature. The dry protein was resuspended in acetone, transferred to a thick-walled glass tube, and homogenized using a motor-driven Teflon pestle. The protein dispersion was deposited on filter paper (Whatman No. 50) using the Tracerlab E8A filtration apparatus. The activity of the samples was counted using a thin window Geiger-Müller tube and scaling circuit. Self-absorption corrections were made by the equational method of Schweitzer and Stein (6), using an experimentally determined value of b equal to 7.0.

Nucleic Acid Synthesis.—In measuring glycine-1-C^{14} uptake into nucleic acid purines (Table XII) three procedures were used. In all cases the incubation mixture consisted of 6.0 ml. of nuclear suspension, 3 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 7.3), 2.4 ml. of 0.1 M glucose containing 6.25 mg. NaCl per ml.,

and 0.6 ml. of H_2O containing 0.6 mg. glycine-1-C^{14}. The flasks were shaken at 37° for 60 minutes, at which time uptake was stopped by adding an equal volume of 10 per cent TCA. The suspensions were centrifuged, and the nuclei were washed once with cold 5 per cent TCA and once with cold 2 per cent perchloric acid. Lipides were then removed by washing once with hot ethanol, and once with warm 2:2:1 ethanol-ether-chloroform mixture.

1. In the first procedure, the total nucleic acid was solubilized by treating the residue in 1 N KOH for 16 hours. The suspension was then brought to pH 7.8, (using 20 per cent $HClO_4$), cooled, and centrifuged. The supernate was placed on a starch block and subjected to electrophoresis, following the procedure of Kunkel and Slater (33). The block was then cut into segments which were extracted with water. The location and amount of the nucleic acid were easily determined by the E_{260}. The eluates containing nucleic acid were lyophilized. The lyophilized material was redissolved and again subjected to electrophoresis. The nucleic acids were eluted with water and lyophilized. The dry powder was homogenized in acetone and plated for counting.

2. In a second procedure, the nucleotides were separated on an ion exchange resin. The acid-washed, lipid-free nuclei were extracted with 30 ml. of 10 per cent $HClO_4$ at 70°C. for 20 minutes. The pH was adjusted to 6.7 with 6 N KOH and the suspension was centrifuged in the cold. The supernate was passed through a column of dowex 1–formate (34), and the column was washed with water. The nucleotides were eluted with 4 N formic acid containing 1 M ammonium formate. The eluate was then lyophilized. The radioactivity of the nucleotides was determined in the usual way.

3. In the third procedure, the purines were separated on a dowex-50 column. The acid-washed, lipid-free nuclei were treated with 20 ml. of 5 per cent TCA at 90° for 15 minutes and centrifuged. The supernate was repeatedly shaken with ether to remove the TCA, and concentrated HCl was added to bring the final concentration to 1 N. The solution was placed in a boiling water bath for 1 hour, cooled, and the acid concentration brought to 2 N with HCl. The bases were then placed on dowex 50 (H^+ form) and eluted following the method described by Cohn (35). The eluates were lyophilized and the specific activity of the mixed purines determined in the usual way.

SUMMARY

1. Nuclei prepared from calf thymus tissue in a sucrose medium actively incorporate labelled amino acids into their proteins. This is an aerobic process which is dependent on nuclear oxidative phosphorylation.

2. Evidence is presented to show that the uptake of amino acids represents nuclear protein synthesis.

3. The deoxyribonucleic acid of the nucleus plays a role in amino acid incorporation. Protein synthesis virtually ceases when the DNA is removed from the nucleus, and uptake resumes when the DNA is restored.

4. In the essential mechanism of amino acid incorporation, the role of the DNA can be filled by denatured or partially degraded DNA, by DNAs from

other tissues, and even by RNA. Purine and pyrimidine bases, monoribonucleotides, and certain dinucleotides are unable to substitute for DNA in this system.

5. When the proteins of the nucleus are fractionated and classified according to their specific activities, one finds the histones to be relatively inert. The protein fraction most closely associated with the DNA has a very high activity. A readily extractable ribonucleoprotein complex is also extremely active, and it is tempting to speculate that this may be an intermediary in nucleocytoplasmic interaction.

6. The isolated nucleus can incorporate glycine into nucleic acid purines, and orotic acid into the pyrimidines of its RNA. Orotic acid uptake into nuclear RNA requires the presence of the DNA.

7. The synthesis of ribonucleic acid can be inhibited at any time by a benzimidazole riboside (DRB) (which also retards influenza virus multiplication (11)).

8. The incorporation of amino acids into nuclear proteins seems to require a preliminary activation of the nucleus. This can be inhibited by the same benzimidazole derivative (DRB) which interferes with RNA synthesis, provided that the inhibitor is present at the outset of the incubation. DRB added 30 minutes later has no effect on nuclear protein synthesis. These results suggest that the activation of the nucleus so that it actively incorporates amino acids into its proteins requires a preliminary synthesis of ribonucleic acid.

9. Together with earlier observations (27, 28) on the incorporation of amino acids by cytoplasmic particulates, these results show that protein synthesis can occur in both nucleus and cytoplasm.

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