sion (33) is an infinitesimal of the first order. Thus $R(\phi, \omega)$ is an infinitesimal of the first order, and, as $\omega \to 0$, the quantities $1 - R(\phi, \omega)$ must tend to unity. On the other hand, the values of the Chandrasekhar-Münch-Limber formulas (5), (6), and (7) ascribed to $1 - R(\phi, \omega)$ are independent of ω and less than unity. This completes the proof of the assertion regarding Limber's method of estimation made in the Introduction.

Incidentally, the general property that $R(\phi, \omega)$ must tend to zero as $\omega \to 0$ is implied by formula (86) of the theory of clustering of galaxies published earlier.[6] For small values of ω, this property dominates the value of $R(\phi, \omega)$ and makes this statistic not very sensitive to properties of the distribution of galaxies in space. The authors wish to call attention to the fact that the allied concept of quasi-correlation is free from this defect.

The other statistic mentioned in the Introduction, which is used by both Limber and Mrs. Rubin, is the quotient $(\overline{N^2}(\omega) - [\bar{N}(\omega)]^2)/[\bar{N}(\omega)]^2$. Here, again, the theoretical expression implied by formulas (5), (6), and (7) is independent of ω. On the other hand, as will be seen from formula (12), the mere fact that $N(\omega)$ is an integer implies that, if images of galaxies are counted in smaller and smaller subdivisions ω of the photographic plate, then the resulting empirical values of the statistic concerned will tend to infinity.

It will be seen that, while in its original form the theory of fluctuations is contradictory to the theory of clustering—for example, with respect to properties of $R(\phi, \omega)$—the reformulation of the theory of fluctuations makes it consistent with the theory of clustering. In fact, the basic concepts of the theory of fluctuations $\bar{p}$, β^2, and $\Gamma(\xi)$, defined by formulas (10), (18), and (20), are easily expressible in terms of the basic concepts of the theory of clustering.

* Prepared with the partial support of the Office of Naval Research.

[1] J. Neyman and E. L. Scott, submitted to the *Astron. J.*

[2] D. N. Limber, *Astrophys. J.*, **117**, 134–144, 1953.

[3] D. N. Limber, *Astrophys. J.*, **119**, 651–681, 1954.

[4] S. Chandrasekhar and G. Münch, *Astrophys. J.*, **115**, 103–123, 1952.

[5] V. C. Rubin, these PROCEEDINGS, **40**, 541–549, 1954.

[6] J. Neyman and E. L. Scott, *Astrophys. J.*, **116**, 144–163, 1952.

AMINO ACID INCORPORATION BY ISOLATED THYMUS NUCLEI. I. THE ROLE OF DESOXYRIBONUCLEIC ACID IN PROTEIN SYNTHESIS

BY V. G. ALLFREY

ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Communicated by A. E. Mirsky, July 23, 1954

Previous investigations in this laboratory have shown that cell nuclei isolated from the calf thymus by differential centrifugation in sucrose solutions are equivalent in total protein content and in enzymatic composition to thymus nuclei isolated in nonaqueous media.[1] The latter nuclei are selected as a standard of comparison because only nonaqueous isolation procedures preclude a loss of nuclear protein or an exchange of soluble proteins between nucleus and cytoplasm.[1, 2] As

far as is known at present, only thymus nuclei can be isolated in sucrose solutions without a loss or exchange of the soluble nuclear proteins. Evidence has been presented to show that this ability to retain soluble proteins is not due to the "impermeability" of the nuclear membrane.[1] "Sucrose" nuclei prepared from other tissues, notably calf and rat liver, are grossly deficient in protein and are modified considerably in their enzymatic compositions. It might also be mentioned that nuclei prepared in sucrose from liver, pancreas, or kidney tissue do not meet the high standard of purity so readily achieved in preparations from the thymus but usually show appreciable contamination by whole cells, fiber, and cytoplasmic debris.

The purity and the apparent enzymatic intactness of the thymus nuclei after isolation in sucrose suggests that such nuclei may be applied to in vitro studies of nuclear metabolism. The experiments described below are concerned with the ability of isolated thymus nuclei to incorporate C^{14}-alanine into nuclear protein. It has been found that an appreciable amino acid uptake can take place in an in vitro system and that this uptake is dependent upon an energy source supplied in the incubation medium.

It has also been found that the uptake of C^{14}-alanine into nuclear protein requires that nuclear desoxyribonucleic acid (DNA) remain intact; the incorporation is nearly abolished by treatment of the nuclei with crystalline desoxyribonuclease. In contrast, ribonuclease, which has been shown to block protein synthesis in isolated cytoplasmic particulates,[3] has no such effect on isolated thymus nuclei.

Preparation of Nuclei.—Fifty grams of fresh calf thymus tissue was finely minced with scissors and placed in a Waring Blendor with 50 ml. of cold 0.5 *M* sucrose solution and 400 ml. of cold 0.25 *M* sucrose–0.0018 *M* $CaCl_2$ solution.[4] The tissue was gently homogenized by running the blendor at 35 volts for 4 minutes. (All operations were performed at 2° C.) The resulting homogenate was filtered through a double layer of gauze (Johnson and Johnson Type I) and then through a single thickness of flannelette. The filtrate was centrifuged at 2,000 rpm (700 $\times$ g) for 10 minutes, and the supernate was discarded. The sediment was resuspended in 90 ml. of 0.25 *M* sucrose–0.0018 *M* $CaCl_2$, transferred to a 100-ml. cylinder, and allowed to settle for 10 minutes. The supernate was carefully decanted through a double thickness of gauze, and the clumps of nuclei, fiber, and whole cells which remain at the base of the cylinder were discarded. The supernate was centrifuged at 2,000 rpm for 10 minutes to sediment the nuclei. The sediments were pooled and washed with about 50 ml. of 0.25 *M* sucrose–0.0018 *M* $CaCl_2$ solution. The washings were discarded, and the nuclei were finally suspended in 25 ml. of 0.25 *M* sucrose–0.0018 *M* $CaCl_2$. Nuclei so prepared were well formed microscopically, and contamination by whole cells or cytoplasmic debris was negligible. The final suspension contained about 25 mg. of nuclei per milliliter.

Incubation Procedure.—Aliquots of the nuclear suspension were incubated aerobically at 37° C. in an isotonic phosphate-sucrose buffer at pH 7.4, in the presence of alanine-1-C^{14} and other metabolites, as described in detail below. The metabolites used, the conditions of the incubation, and the final preparation of protein are essentially those recommended by Siekevitz in incorporation studies of cytoplasmic particulates.[5]

Each incubation vessel contained 1.0 ml. of nuclear suspension, 0.5 ml. of phosphate-sucrose buffer (3 parts of 0.1 *M* potassium phosphate buffer, pH 7.4, to 7

parts of 0.25 M sucrose solution), 3.2 μM of adenosine-5-phosphoric acid, and 10 μM of $MgCl_2$. All flasks contained 40 μM of α-ketoglutaric acid, except for those cases where it was desired to demonstrate that the extent of amino acid incorporation is greatly diminished in the absence of an energy-yielding substrate. To each flask, 0.1 mg. of alanine-1-C^{14} (1.0 mc/mM) was added. The final volume of solution was 2.0 ml.; the final pH was 7.4.

The flasks were then immersed in a 37° water bath and shaken at 120 cycles per minute for 1 hour. All experiments were performed in quadruplicate, the contents of two vessels being pooled following incubation for preparation of the nuclear protein.

To investigate the effects of ribonuclease and desoxyribonuclease upon this system, flasks containing nuclei and all metabolites (except C^{14}-alanine) were preincubated at 37° for 15 minutes in the presence of either crystalline ribonuclease or crystalline desoxyribonuclease. (These enzymes were generously supplied by Dr. Moses Kunitz, of the Rockefeller Institute.) In the corresponding "nuclease control" experiments, the nuclei were incubated in the absence of the enzyme. Radioactive alanine was then added and the incubation carried out for 1 hour as before.

In all cases "control" experiments were performed to ascertain the extent to which C^{14}-alanine is adsorbed by nuclear proteins. The procedure in the controls was to add the full amount of alanine-1-C^{14} to the suspension of nuclei plus metabolites. The mixture was stirred, and an equal volume of cold 10 per cent trichloroacetic acid was added immediately. The resulting precipitate was then treated as described below for the preparation of the nuclear protein.

Preparation of Nuclear Protein.—Following incubation, the contents of duplicate vessels were pooled. Two milliliters of water were added, followed by 6.0 ml. of cold 10 per cent trichloroacetic acid (TCA). The suspension was centrifuged, and the precipitate was washed twice with 30 volumes of 5 per cent TCA. The precipitate was then suspended in 5 per cent TCA, brought to 85° C. for 15 minutes (to extract the nucleic acids), and then centrifuged. The sediment was washed once more with 5 per cent TCA. To remove lipids 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 E 8-A filtration apparatus. The activity of the samples was counted by using a thin-mica-window Geiger-Müller tube and scaling circuit. The area of all samples was 2.69 $cm.^2$ The counting error was approximately 3 per cent. 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.

Results.—Incorporation data are summarized in Table 1 for three series of experiments on thymus nuclei isolated in sucrose. The activity of the nuclear protein (in counts per minute per milligram) is given in column 1 of the table. The "corrected activity" (i.e., the measured activity minus the activity of the adsorption "controls" is listed in column 2.

TABLE 1*

ALANINE-1-C^{14} INCORPORATION BY ISOLATED CALF THYMUS NUCLEI

	Conditions of Experiment	C^{14} Activity of Nuclear Protein (cpm/mg Protein) Total Activity (1)	Corrected Activity (2)
A.	Adsorption "control"	62	0
	Nuclei + α-ketoglutarate	209	147
	Nuclei + α-ketoglutarate + ATP	180	118
	Nuclei − α-ketoglutarate	118	56
	Ribonuclease-treated nuclei	197	135
B.	Adsorption "control"	118	0
	Nuclei + α-ketoglutarate	265	147
	Nuclei − α-ketoglutarate	204	86
	Ribonuclease-treated nuclei	228	110
	Desoxyribonuclease-treated nuclei [enzyme acting for 75 minutes]	131	13
	Nuclease "control"	202	84
C.	Adsorption "control"	55	0
	Nuclei + α-ketoglutarate	161	106
	Nuclei − α-ketoglutarate	80	25
	Desoxyribonuclease-treated nuclei [enzyme acting for 15 minutes]	126	71
	DNAase-treated nuclei + added DNA	158	103
	Nuclease "control"	174	119

* In vitro incorporation of radiocarbon into the protein of calf thymus nuclei isolated in sucrose and then incubated in the presence of C^{14}-alanine and added metabolites. Each incubation vessel contained 1.0 ml. of nuclear suspension (25 mg. nuclei), buffered in isotonic phosphate-sucrose at pH 7.4, together with 3.2 μM of adenosine-5-phosphoric acid and 10 μM of $MgCl_2$. All flasks contained 40 μM of α-ketoglutaric acid except for experiments listed in the table as "Nuclei − α-ketoglutarate." One-tenth milligram of alanine-1-C^{14} (1.0 mc/mM) was then added, and the flasks were shaken at 37° for 60 minutes. In the adsorption "control" the reaction was stopped at $t = 0$ by the addition of an equal volume of 10 per cent trichloroacetic acid. The data in column 1 give the total activity in counts per minute per milligram of protein. The corrected activities in column 2 represent the difference between the total activity and the C^{14} uptake of the absorption "control."

There are five main points which emerge after consideration of the data.

1. The uptake of C^{14}-alanine into the protein of isolated thymus nuclei is appreciable. The incorporations observed (106–147 cpm/mg in the presence of α-ketoglutarate) are in sharp contrast to the low uptakes previously reported for in vitro systems of rat liver[5] and swine kidney sucrose nuclei.[7] It has also been observed that calf thymus nuclei isolated in nonaqueous media by a modified Behrens' procedure[2] are unable to incorporate C^{14}-alanine under these experimental conditions.

2. The incorporation of labeled amino acid into nuclear protein requires a source of energy. The incorporations observed in the absence of added α-ketoglutarate are only 24–58 per cent of those obtained in the presence of the substrate. The addition of ATP to systems already containing adenosine-5-phosphate, $MgCl_2$, and α-ketoglutarate did not lead to further increases in amino acid uptake.

3. The incorporation of C^{14}-alanine is not inhibited by preincubation of the nuclei with ribonuclease. The failure of ribonuclease to block amino acid incorporation by the isolated nucleus is in sharp contrast to its effect on isolated cytoplasmic particulates.[3] C^{14}-alanine uptake into "microsome" proteins is greatly diminished in the presence of this enzyme. It should be noted, however, that although desoxyribonuclease has actually been demonstrated to enter the nucleus and to break down its DNA,[1] no such evidence has yet been presented to show that ribonuclease acts upon the RNA of the cell nucleus.

4. Preincubation of the thymus nucleus with desoxyribonuclease destroys or impairs its capacity to incorporate radioactive amino acid. Under the conditions of these experiments, preincubation for 15 minutes with crystalline desoxyribonu-

clease (subsequent enzymic action being inhibited by the addition of 20 μM of trisodium citrate) led to a 40 per cent reduction in C^{14} uptake. When desoxyribonuclease was allowed to act throughout the whole of the incubation period (75 minutes total), the C^{14} uptake was only 15 per cent of that observed in control experiments.

5. The addition of supplementary calf thymus desoxyribonucleic acid (DNA) to nuclei previously treated with desoxyribonuclease results in higher amino acid incorporations than are observed in the absence of added DNA. This result is reminiscent of the recent experiments of Gale[8] on amino acid incorporation by broken bacterial cells, in which it was found that added DNA (as well as RNA and a purine-pyrimidine mixture) could restore the protein synthetic capacity of nuclease-treated cells.

In summary, the experiments described above have shown that isolated calf thymus nuclei can incorporate C^{14}-alanine into nuclear protein and that this incorporation requires that the desoxyribonucleic acid of the nucleus remain intact.

[1] H. Stern and A. E. Mirsky, *J. Gen. Physiol.*, **37**, 177, 1953.
[2] V. Allfrey, H. Stern, A. E. Mirsky, and H. Saetren, *J. Gen. Physiol.*, **35**, 529, 1952.
[3] V. Allfrey, M. M. Daly, and A. E. Mirsky, *J. Gen. Physiol.*, **37**, 157, 1953.
[4] R. M. Schneider and M. L. Petermann, *Cancer Research*, **10**, 751, 1950.
[5] P. Siekevitz, *J. Biol. Chem.*, **195**, 549, 1952.
[6] G. K. Schweitzer and B. R. Stein, *Nucleonics*, **7**, 65, 1950.
[7] K. Lang, H. Lang, G. Siebert, and S. Lucius, *Biochem. Z.*, **324**, 217, 1953
[8] E. F. Gale and J. P. Folkes, *Nature*, **173**, 1223, 1954.

---

*STUDIES ON UNBALANCED GROWTH IN ESCHERICHIA COLI**

BY SEYMOUR S. COHEN AND HAZEL D. BARNER

CHILDREN'S HOSPITAL OF PHILADELPHIA (DEPARTMENT OF PEDIATRICS) AND DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY OF THE UNIVERSITY OF PENNSYLVANIA SCHOOL OF MEDICINE

Communicated by David Goddard, August 11, 1954

The discovery of a unique viral constituent, 5-hydroxymethylcytosine,[1] has led this laboratory to metabolic studies on pyrimidines. Among the approaches employed have been nutritional investigations with mutants of *Escherichia coli* requiring specific pyrimidines. One of these organisms was a thymine-requiring strain, designated 15_{T^-}. It was found that, on infection with T2 bacteriophage in the absence of thymine, the infected 15_{T^-} produced as much virus and DNA as if thymine had been added to the medium.[2] More detailed investigation revealed that the infected cell synthesized thymine in considerable quantity as a component essential to virus deoxyribonucleic acid (DNA).[3] Indeed, the only nucleic acid pyrimidines synthesized after infection were thymine and 5-hydroxymethylcytosine, neither of which appeared to be synthesized in appreciable amounts before infection. The existence of this phenomenon led to a study of other properties of strain 15_{T^-}.

Consequences of Thymine Deficiency.—It has been observed that, in a synthetic medium lacking thymine, the organism irreversibly loses the power to form colonies ("dies") at the rate of about 90 per cent per division time. This type of steriliza-