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*AMINO ACID ACTIVATION AND TRANSFER TO RIBONUCLEIC ACIDS IN THE CELL NUCLEUS**

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The *in vitro* incorporation of amino acids into the proteins of calf thymus nuclei (isolated in isotonic sucrose) has been shown to be dependent upon the presence of ATP.[1, 2] In isolated nuclei ATP synthesis depends on an aerobic phosphorylating system which differs from that of mitochondria in being insensitive to various mitochondrial inhibitors (carbon monoxide, methylene blue, and calcium ions).

All agents which have been found to block nuclear generation of ATP also block amino acid incorporation into proteins.[1, 2]

Zamecnik and his co-workers[3, 4] and Siekevitz[5] have described energy dependent, cell-free cytoplasmic systems which incorporate amino acids into protein. The first step in the process is considered to be an enzymatic activation of the carboxyl groups of amino acids.[6] This reaction requires ATP. The amino acyladenylate compounds which are formed remain bound to the enzyme surface.[3]

$$\text{ATP} + \text{AA} + \text{Enzyme} = \text{Enzyme}-\text{AMP}-\text{AA} + \text{PP}$$

Subsequently the activated amino acid is transferred to the 2′ or 3′ position of the terminal adenine nucleotide of soluble ribonucleic acids by the activating enzyme.[7, 8, 4]

$$\text{E}-\text{AMP}-\text{AA} + \text{sRNA} = \text{sRNA}-\text{AA} + \text{AMP} + \text{E}$$

Enzymes catalyzing these reactions, the pH 5 enzymes, have been prepared from extracts of a variety of animal,[3, 9] plant, [10, 12] and bacterial cells[13, 14] by precipitation (from the soluble components of the cell) at pH 5. In cytoplasmic protein synthesis, incorporation of the amino acid into polypeptides takes place later, and requires the presence of the microsomal fraction.[15]

The present paper is concerned with a similar series of reactions in cell nuclei.

Evidence will be presented to show that pH 5 activating enzymes are present in significant amounts in cell nuclei prepared in both aqueous and nonaqueous media. During the process of incorporation of radioactive amino acids by isolated calf thymus nuclei, a part of the nuclear RNA (which can be isolated by the phenol method[16]) becomes labeled with the amino acid.

Materials and Methods.—Fresh calf thymus nuclei were prepared in isotonic sucrose with 0.0030 *M* $CaCl_2$ by the method previously described.[1] After washing twice with sucrose solution, pH 5 enzymes were prepared from the nuclear pellet following the procedure used by Hoagland *et al.* for rat liver.[3] The precipitate formed at pH 5.2 was dissolved in 0.1 *M* tris buffer at pH 7.6. In most preparations this solution was dialyzed against 2 changes of 100 volumes each of this buffer for 3 hr to remove free amino acids. (All steps were carried out at 0° to 2°C.) The pH 5 enzymes from whole thymus tissue were prepared similarly using well minced tissue rather than the nuclear pellet as starting material.

Nonaqueous nuclei from calf thymus and chicken kidney and their tissue "controls" were isolated as described by Allfrey *et al.*[17] The pH 5 enzymes from the nonaqueous material were prepared and assayed in the same way as described for fresh material.

Amino acid-dependent exchange of P^{32}-pyrophosphate with ATP was used to determine the amino acid activating activity of the pH 5 enzyme fraction. P^{32}-pyrophosphate was prepared by pyrolysis. The ATP^{32} formed was adsorbed on charcoal and its radioactivity was determined by the procedure of Crane and Lipmann.[18] Phosphorus analyses were done using Allen's method.[19] Protein concentration was measured by the method of Lowry[20] or by direct weighing.

Two μmoles each of the 12 L-amino acids used by Hoagland *et al.*[3] were employed for most assays. These were leucine, isoleucine, valine, glycine, threonine, histidine, phenylalanine, tryptophan, serine, alanine, arginine, and lysine. DNAase and RNAase were supplied by Worthington Biochemical Corp.; Na_2ATP was

from Sigma Chemical Co.; Parke and Davis Chloramphenicol was used. The pH of all solutions was adjusted to 7.6 before use.

RNA was prepared by the phenol method of Kirby[16] using freshly distilled phenol. The RNA was washed 4 times with cold 2 per cent perchloric acid, 3:1 ethanol:

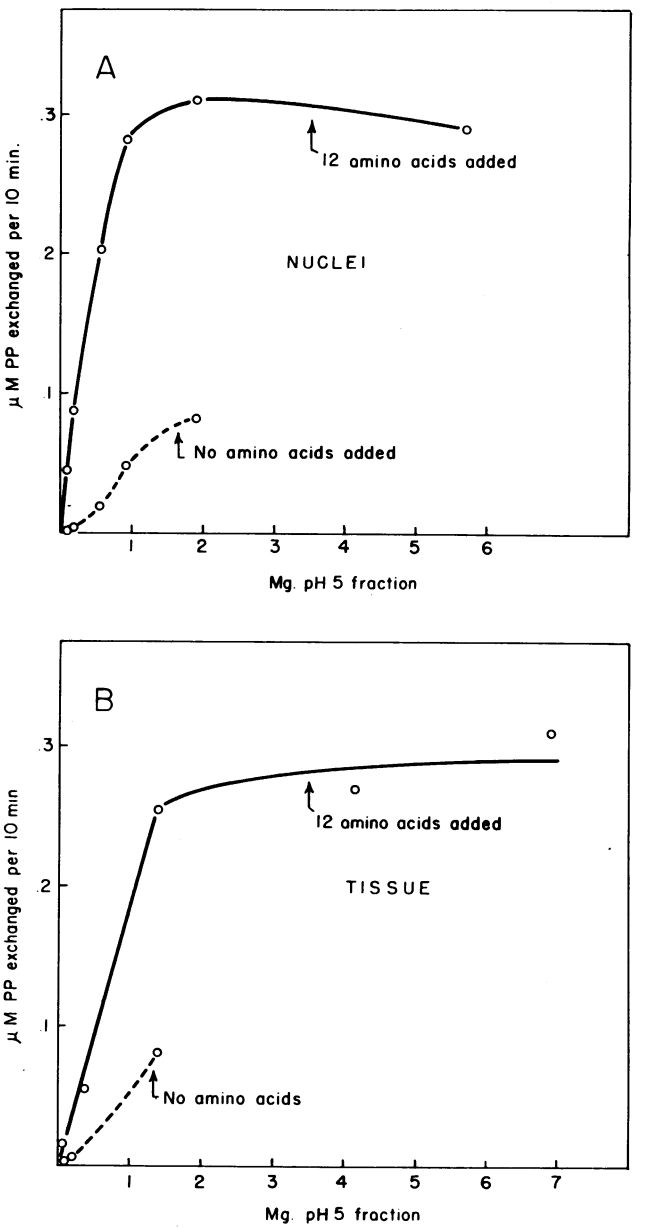

FIG. 1.—The relationship between ATP P^{32}-pyrophosphate exchange and concentration of the pH 5 enzyme fraction from fresh calf thymus nuclei (A) and tissue (B).

Each flask contained 5 μmoles of ATP, 1 μmole of P^{32}-pyrophosphate and the indicated amounts of pH 5 enzymes in 0.1 *M* "tris" buffer at pH 7.6. Two μmoles each of the 12 L-amino acids listed in the text were added. The solution contained 5 μmoles $MgCl_2$ and 10 μmoles KF. The final volume was 1.0 ml. Incubations were carried out in air at 37°C with gentle agitation for 10 min. The exchange reaction was stopped by adding 2.0 ml cold 10 per cent trichloroacetic acid.

ether, and ether to insure complete removal of free amino acids before determining its radioactivity. Protein was prepared for counting as described by Allfrey *et al.*[1] Radioactivity was measured in a thin-window gas flow counter and the counts were corrected for self absorption by the method of Schweitzer and Stein.[21]

Results.—Extracts of thymus nuclei and whole tissue catalyze the ATP P^{32}-pyrophosphate exchange reaction. Figure 1 illustrates the amino acid-dependent nature of the exchange. When the pH 5 enzyme fraction is prepared as described, but not dialyzed, some exchange is observed in the absence of added amino acids. This is due presumably to the presence of residual free amino acids. Dialysis for 3 hr reduces this residual exchange to less than 15 per cent of that observed in the presence of a mixture of 12 amino acids. A similar effect can be obtained by treatment with Norit A, following the procedure described by Clark.[12]

TABLE 1

EFFECT OF AMINO ACIDS ON PYROPHOSPHATE-ATP EXCHANGE

Amino Acids Promoting Exchange	Amino Acids Without Effect in This System
L-Cysteine	D-Amino acids
L-Histidine	L-Alanine
L-Isoleucine	L-Arginine
L-Lysine	L-Aspartic acid
L-Leucine	L-Glutamic acid
L-Methionine	Glycine
L-Proline	L-Phenylalanine
L-Serine	
L-Threonine	
L-Tryptophan	
L-Tyrosine	
L-Valine	

The exchange occurs with mixtures of amino acids or with amino acids tested separately. Twelve amino acids which promote PP^{32} are listed in the first column of Table 1. The nuclear pH 5 fraction from fresh calf thymus does not promote exchange reactions in the presence of D-amino acids. To date, no stimulation has been observed for the other 6 amino acids listed in Table 1.

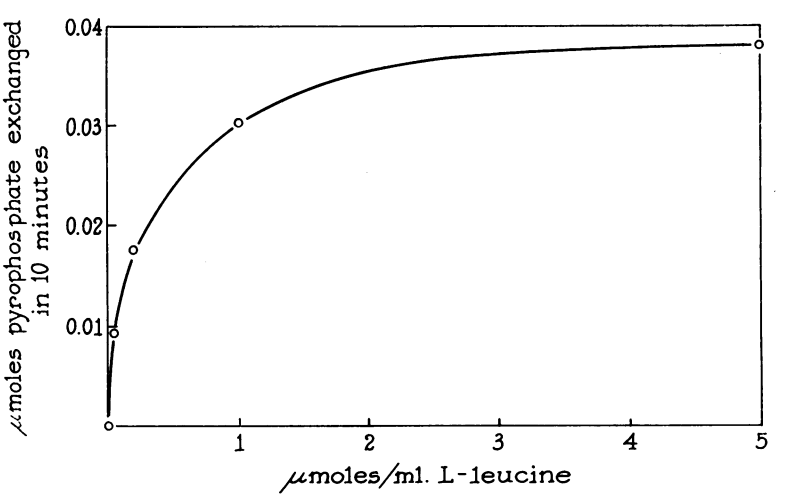

FIG. 2.—The relationship between P^{32}-pyrophosphate-ATP exchange and the amount of L-leucine added to the pH 5 enzyme fraction of calf thymus nuclei. Incubation conditions were the same as described in the legend to Figure 1. One milligram pH 5 fraction was added.

The rate of exchange depends on amino acid concentration. Figure 2 illustrates this dependence for L-leucine.

The evidence for nuclear localization: The occurrence of amino acid activating

enzymes in nuclei isolated in *aqueous* media is not in itself a proof of nuclear localization. Since these enzymes are water-soluble, the possibility must be considered that their presence in nuclei is the result of adsorption of cytoplasmic enzymes during the process of isolation.

The transfer or exchange of enzymes between nucleus and cytoplasm in the course of isolation in *aqueous media* is well documented.[22] However, a proof of nuclear localization can be obtained from a study of nuclei isolated in *nonaqueous media* by the Behrens procedure[23] or its modifications.[24, 17] In this method the tissue is rapidly frozen and lyophilized. This prevents any loss or exchange of soluble cell components. After grinding, the nuclei are isolated in mixtures of aqueous solvents by density gradient centrifugation.

Nuclear preparations of high purity can be obtained. Particularly pure preparations have been isolated from chicken kidney and calf thymus.[25]

These preparations have been tested for their content of amino acid activating enzymes. The results are summarized in Table 2. For purposes of comparison,

TABLE 2

	Column 1 Activity* per mg pH 5 Fraction	Column 2 Activity* per 100 mg Starting Material	Column 3 Activity* per 100 mg Whole Cells		Column 4 Per Cent of Total Activity in the Nucleus
			Nucleus	Cells	
Behrens calf thymus nuclei	0.050	0.082	0.050	...	70.4%
Behrens calf thymus tissue	0.048	0.071	...	0.071	...
Behrens chicken kidney nuclei	0.051	0.120	0.018	...	7.6%
Behrens chicken kidney tissue	0.080	0.236	...	0.236	...
Fresh calf thymus nuclei	0.46	0.570	0.348	...	62.5%
Fresh calf thymus tissue	0.22	0.557	...	0.557	...

* μmoles pyrophosphate exchanged in 10 min. Incubation conditions as in Figure 1.

the activities oberved in extracts of isolated nuclei have been compared with those of whole tissue extracts. (Tissue "controls" were prepared by lyophilizing and treating with the same organic solvents used in the isolation of nuclei.[17]) The pH 5 enzymes were prepared from nuclei and whole tissue as described in *Methods*.

The first column in Table 2 lists the specific activities of the activating enzyme fractions (as μmoles of pyrophosphate exchanged per 10 min per mg of pH 5 fraction). A mixture of 12 amino acids (see *Methods*) was present during the assay.

The second column indicates the total exchange activity contained in the pH 5 fraction from 100 mg starting material (either isolated nuclei or whole tissue). It can be seen that 100 mg of thymus nuclei contain slightly more activating enzyme than the equivalent weight of whole tissue. In kidney nuclei the total nuclear activity is about 51 per cent of the activity of the tissue control.

What per cent of the amino acid activating activity of the cell occurs in the nucleus?

The answer to this question can be obtained by comparing the activity of an equal number of nuclei and whole cells. Since in the thymus the nucleus comprises

61 per cent of the mass of the cell,[17] the figures for total activity of the nuclei listed in column 2 of the table can be multiplied by the factor 61/100 to give the total enzymatic activity in the nuclei of 100 mg of cells. The ratio of this figure to the activity in 100 mg of whole cells represents the fraction of the total amino acid activating activity of the whole cell that is localized in the nucleus. These ratios are given in column 4 of the table. In thymus the nuclei contain about 70 per cent of the total activity of the cell; in kidney the nuclei comprise only 15 per cent of the mass of the cell[17] and contain only about 8 per cent of the total activity.

It should be stressed, however, that other tests for the purity of chicken kidney nuclei isolated in nonaqueous media indicate that cytoplasmic contamination cannot account for the presence of the amino acid activating enzymes in the isolated nucleus. For example, the soluble enzyme, catalase, is completely absent from such nuclei although it is abundant in the cytoplasm. Less than 1 per cent of the total arginase activity of the cell occurs in the kidney nuclei. This is a significant test for purity because arginase occurs in extremely high concentrations in the cytoplasm of chicken kidney cells.[25]

In calf thymus nuclei isolated in nonaqueous media the presence of 70 per cent of the total activity of the cell in the nuclei cannot be explained in terms of cytoplasmic contamination. The purity of these nuclei has been established in many ways[26, 33] ranging from electron microscopy to immunological tests for purity.

The occurrence of amino acid activating enzymes in calf thymus nuclei isolated in isotonic sucrose solutions is another instance of the capacity of these nuclei to withstand isolation in this aqueous medium without a loss of water-soluble components.[22]

The specific activity of the pH 5 fraction from thymus "sucrose" nuclei is also listed in Table 2. A comparison of the total nuclear activity with the total activity of the cell shows that about 63 per cent of the amino acid activating activity of the cell is found in the nucleus. This is in good agreement with the results on nuclei isolated in nonaqueous media.

Is amino acid transferred to DNA in the nucleus? Incorporation of amino acids by calf thymus nuclei is blocked by removal of the DNA by DNAase.[1] Amino acid uptake can be restored by adding back DNA, but even RNA or synthetic polyanions (such as polyethylene sulfonate) can restore C^{14} uptake.[2] This makes it unlikely that DNA is directly involved in protein synthesis as an intermediate acceptor of activated amino acids in the nucleus. However, to test this possibility thymus nuclei isolated in 0.25 *M* sucrose were incubated with DL-leucine-2-C^{14} as described previously.[1] The nuclei were washed 3 times at 2°C with 0.9 per cent NaCl containing 0.01 *M* sodium citrate and the DNA isolated by the sodium dodecyl sulfate method B of Marko and Butler.[27] Parallel incubations were run to determine the amino acid incorporation into the total proteins of the nuclei. After 60 minutes incubation at 37°C the protein contained 381 cpm/mg while the DNA contained only 11 cpm/mg. Protein analysis of the DNA[(20)] showed 2 per cent contamination by protein. It therefore seems likely that contaminating protein can account for all, or nearly all, of the counts associated with DNA, and there is thus no evidence for a transfer of leucine-C^{14} to the DNA of the nucleus.

The inhibition of nuclear protein synthesis by chloramphenicol: Breitman and Webster[28] have shown that a high concentration (0.0067 *M*) of the antibiotic

chloramphenicol blocks amino acid incorporation in isolated calf thymus nuclei. We have confirmed this and have also tested to see whether ATP synthesis in nuclei is impaired by chloramphenicol. A comparison of ATP synthesis with and without chloramphenicol present showed that no inhibition occurred; on the contrary, slightly more ATP was present in nuclei exposed to the antibiotic.

Since chloramphenicol does not affect ATP synthesis, the question arises of its effects on amino acid activation. In a study of tryptophan activating enzyme[29] Acs and Lipmann found no effect of chloramphenicol on pyrophosphate-ATP exchange.[30] The nuclear pH 5 fraction is similarly unaffected. Furthermore, the results described below indicate that chloramphenicol does not block amino acid transfer to the RNA of isolated thymus nuclei. This indicates that the antibiotic acts at a later stage of protein synthesis.

Transfer of activated C^{14} amino acids to nuclear RNA: To test the possible involvement of nuclear RNA in protein synthesis within the nucleus, isolated calf thymus nuclei were incubated in the medium previously described (Table 3) in

TABLE 3

LABELING OF NUCLEAR RNA BY AMINO ACID

	CPM/mg
DL-Leucine-1-C^{14} (1.09 mcuries/mmole)	
Protein:	
Control	430
0.0067 *M* chloramphenicol	6
RNA:	
Control	153
0.0067 *M* chloramphenicol	161
DL-Leucine-1-C^{14} (1.09 mcuries/mmole)	
Protein:	
Control	248
0.0067 *M* chloramphenicol	8
RNA:	
Control	83
0.0067 *M* chloramphenicol	78
DL-Alanine-1-C^{14} (1.92 mcuries/mmole)	
RNA:	
Control	51
0.0067 *M* chloramphenicol	48

Incubation was at 37°C under previously described conditions.[1] After 30 min the nuclear RNA was isolated as described in the text.

the presence of DL-leucine-1-C^{14} or DL-alanine-1-C^{14}. Chloramphenicol was added to some of the flasks to prevent protein synthesis. This procedure permitted the isolation of RNA with no danger of contamination by radioactive protein. After 30 min the nuclei were centrifuged down, the medium soluble material discarded, the nuclei washed twice with 20 volumes of cold incubation mixture containing a 10-fold concentration of the C^{12}-amino acid used and the RNA isolated by the phenol method.[16] RNA was finally precipitated with ethanol, washed four times with cold 2 per cent perchloric acid, with 3:1 ethanol:ether, and with ether and counted. The extent of incorporation into the nuclear proteins, both in the presence and absence of chloramphenicol is shown in Table 3. The table also shows the incorporation of labeled amino acid into RNA. It can be seen that although 0.0067 *M* chloramphenicol inhibits nearly completely the incorporation of amino acid into protein, it has no such effect on the incorporation into RNA.

The linkage between the isolated ribonucleic acid and bound amino acid is

stable in acid and labile in alkali. Upon hydrolysis of the leucine-RNA in 0.005 N NaOH and subsequent chromatography on paper[31] the label is recovered as leucine. Digestion with ribonuclease also separates the radioactivity from the acid precipitable fragments. Current work is in progress to determine whether the amino acid is bound to adenylic acid or to other nucleotides of the RNAase digest.

It is a matter of some interest to know whether the RNA "carriers" in the cell nucleus are large molecules. In preliminary experiments with C^{14} leucine labeled RNA, there have been indications that some of the RNA carrying amino acid is dialyzable. This RNA is still precipitable with perchloric acid. Whether this small RNA occurs in the nucleus as such, or whether it is the result of autolysis during incubation, remains to be determined.

The time course of amino acid uptake into the RNA of the nucleus is given in Figure 3. The upper curve shows the incorporation of leucine-1-C^{14} into total

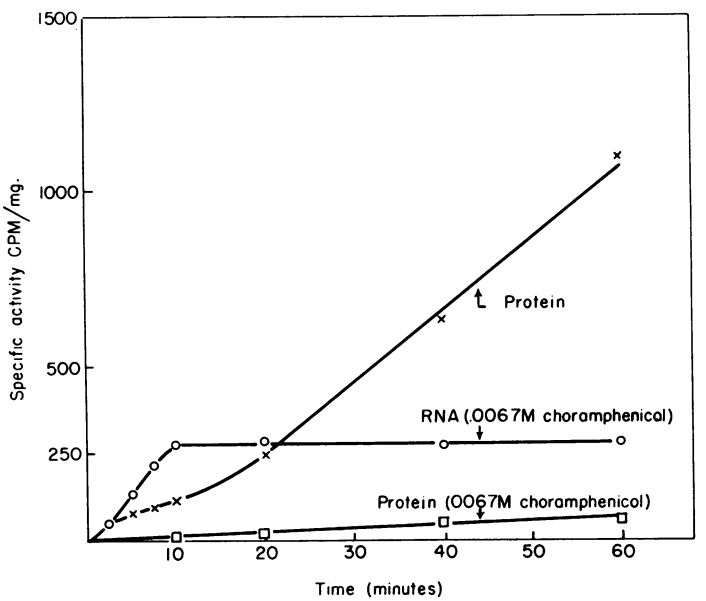

FIG. 3.—The uptake of leucine-1-C^{14} (5.3 mcuries/mmole) into RNA and protein of calf thymus nuclei. Incubation procedures were as previously described[1].

nuclear protein. This curve shows the characteristic lag period observed previously[1]. The middle curve gives the time course of leucine uptake into the isolated nuclear RNA. Chloramphenicol was present in these experiments and under these conditions the uptake into protein is negligible, as shown by the bottom curve in the figure. Although there is a lag in amino acid incorporation into protein, there is no corresponding delay in amino acid uptake into the RNA of the nucleus; this is linear up to 10 min.

These experiments suggest a direct role of nuclear RNA in protein synthesis in the nucleus. This is in accord with earlier results relating RNA to amino acid uptake by thymus nuclei where it was shown that the inhibition of RNA synthesis by the antimetabolite DRB (5,6-dichloro-β-D-ribofuranosylbenzimidazole) results in a subsequent inhibition of amino acid uptake.[1,2]

Which of the ribonucleic acids of the thymus nucleus function as carriers? This has been tested by fractionation of the nuclei after incubation with radioactive amino acids according to Allfrey *et al.*[1] The subsequent isolation of the RNA from the different fractions shows that the readily extractable RNA of the nucleus is most highly labeled with C^{14}-leucine. It is significant that treatment of isolated thymus nuclei with ribonuclease has no effect on protein synthesis[1] and also has no effect on the readily extractable nuclear RNA (while it is in the nucleus).[32] The insoluble "nucleolar" RNA fraction does not appear to function as an amino acid carrier. (It should be mentioned, however, that the isolation of the "nucleolar" RNA requires more time than the preparation of the "soluble" RNA fraction, and some loss of C^{14}-amino acid from "nucleolar" RNA might have occurred.)

It remains to be seen whether other ribonucleic acid fractions in the nucleus participate in amino acid transfer reactions.

Summary.—A pH 5 enzyme fraction catalyzing an amino acid-dependent exchange of pyrophosphate with ATP (a test for amino acid activation) has been found in calf thymus nuclei prepared in both sucrose solutions and in nonaqueous media. Similar activating enzymes occur in chicken kidney nuclei prepared in nonaqueous media.

Twelve L-amino acids have been shown to stimulate pyrophosphate-ATP exchange in the presence of the "pH 5 fraction" from calf thymus nuclei while D-amino acids have no such effect.

DNA isolated from calf thymus nuclei which have been incubated with DL-leucine-2-C^{14} and are actively incorporating amino acids into their proteins contains a negligible amount of labeled amino acid.

RNA isolated from calf thymus nuclei incubated under the same conditions contains a significant amount of labeled amino acid which is rapidly released by dilute alkali or RNAase but not by acid.

Chloramphenicol (0.0067 *M*) inhibits nearly completely the incorporation of amino acid into protein of calf thymus nuclei but has no effect on its incorporation into nuclear RNA.

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*A GENERAL METHOD FOR THE LABELING OF THE ACTIVE SITE OF ANTIBODIES AND ENZYMES**

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Successful labeling of the active site has been dependent upon the particular characteristics of the enzyme system under study. One example is phosphoglucomutase which forms a kinetically stable intermediate.[1-3] Other examples are the esterases, i.e., chymotrypsin and trypsin, in which the serine at the active site reacts much more rapidly with diisopropylfluorophosphate than the other serines in the molecule.[4-8] However, there are many enzymes and proteins of special interest, such as antibodies, whose active sites do not possess any such fortunate chemical properties. In this paper a general method is presented for the labeling of the active site in these cases.

The method was developed from the classic experiments of Hopkins *et al.*[9] and the more recent developments of Cohen *et al.*[7] and Pressman and Sternberger[10] which showed that groups at the active site can be protected from reaction by the prior addition of substrate or competitive inhibitor. The method consists of three basic steps: (1) the treatment of the protein in the presence of substrate with a specific unlabeled reagent until the groups which react with it are saturated; (2) the removal of the substrate; (3) the reaction of the groups at the active site with the same reagent in which a radioactive label has been incorporated.

It is obvious that in order to apply this method of labeling the system under study must fulfill certain requirements. First, the reagent chosen must be capable of