as in MVA, even though an aldehyde had been formed as an intermediate. This possibility was considered unlikely and has now been ruled out by the experiments described later in the paper.

[8] C. H. Shunk, B. O. Linn, J. W. Huff, J. L. Gilfillan, H. R. Skeggs, and K. Folkers, *J. Am. Chem. Soc.*, **79**, 3294, 1957.

[9] H. Daniellson, B. Amdur, and K. Bloch, unpublished work.

[10] B. Vennesland and F. H. Westheimer, in *The Mechanism of Enzyme Action* (Baltimore: Johns Hopkins Press, 1954), p. 357.

[11] If decarboxylation to isopropyl derivatives took place, the two terminal units would remain as CH_2D, but the four internal units would be reconverted into intrachain $—CH_2—$ groups when squalene was formed. Assuming no rate difference between the rupture of C—H and C—D bonds, 4/3 atoms of D would be lost, leaving approximately 5 atoms in squalene.

[12] This discussion has considered transformations involving non-substituted MVA. It has, however, been demonstrated recently that a phosphate ester of MVA is formed from MVA and ATP and that this derivative can be further converted to squalene (T. T. Tchen, *J. Am. Chem. Soc.*, **79**, 6344, 1957). This suggests that one or both hydroxyl groups may be phosphorylated before removal.

[13] It should be pointed out that the formation of the sesquiterpene need not be concerted but could occur in several consecutive steps. For example, the first isoprene molecule, instead of condensing with another isoprene, may interact with an isopentenol phosphate to form geranyl phosphate. The monoterpene derivative could undergo phosphate elimination to form a new carbonium ion for condensation with another C_5 unit. This mechanism is mentioned as one of several possible variations of the general scheme shown in Fig. 3.

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[18] The observation that the succinic acid derived from the central carbon atom of squalene contained deuterium, though only a fraction of an atom, is not in accord with the coupling of two farnesene molecules. On the other hand, if one of the two C_{15} units were farnesol, the succinic acid should contain a minimum of 1 atom of D. The presence of the small amount of D at the two central carbon atoms of squalene remains to be explained.

THE INCORPORATION OF AMINO ACIDS INTO RIBONUCLEIC ACID

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The incorporation of radioactive leucine, valine, and glycine into the ribonucleic acid (RNA) of a soluble enzyme fraction of rat liver has recently been reported by Hoagland *et al.*[1] Ogata and Nohara[2] have studied the incorporation of alanine into RNA. The former authors consider this incorporation to be an intermediate stage in the transfer of labeled amino acids into microsomal protein. This paper describes the fractionation of the crude system for amino acid incorporation into RNA into an activating enzyme fraction and RNA. Both these components are required for incorporation, thus providing a biological assay for functional RNA. Evidence is presented that a specific activating enzyme and a specific RNA are involved in the incorporation of each amino acid.

Methods and Materials.—Amino acid-activating enzyme fraction (pH 5 enzyme) was prepared from guinea-pig liver by a modification of the method of Keller and Zamecnik.[3] This was fractionated with aged calcium phosphate gel.[4] The pH 5 enzyme (10 mg/ml) was treated with one-fifth volume of gel (30 mg/ml) and centrifuged, yielding a supernatant fraction ("gel super"). The precipitated gel was eluted with 2.0 *M* phosphate buffer, pH 8.1, centrifuged, and the eluate dialyzed ("gel eluate"). AS_{30-60} fraction is that fraction of the "gel super" which precipitates between 30 and 60 per cent saturation with ammonium sulfate at pH 7.5. Treatment with ribonuclease (Table 2) involved preincubation with 0.1 μg. of crystalline ribonuclease for 10 minutes at 37°, then dilution with the assay constituents and the usual assay. The low level of ribonuclease used and the dilution minimize ribonuclease action during the assay.

The reaction mixture for incorporation contained: 8 mg. of the pH 5 fraction or the equivalent aliquot; 10 μmoles of potassium adenosine triphosphate (ATP), pH 7.5; 10 μmoles of magnesium chloride; 100 μmoles of tris buffer, pH 7.5; either 0.05 μmoles of uniformly labeled C^{14}-L-leucine (1,100 counts/min/mμmole), 0.025 μmoles of uniformly labeled C^{14}-L-tyrosine (3,000 counts/min/mμmole), or 0.025 μmoles of uniformly labeled C^{14}-L-threonine (2,500 counts/min/mμmole); and water to a final volume of 1.4 ml. The mixture was incubated for 10 minutes at 37°, precipitated with 8 volumes of 3.5 per cent trichloracetic acid, and washed as described by Hoagland *et al.*[1] Inert protein was added before precipitation (when needed) to make the final weight of protein plus RNA counted approximately 10 mg. The pyrophosphate exchange assay for activating enzyme has been described.[5] All samples were counted with a Nuclear "Micromil" window gas-flow counter.

Experiments and Results.—Table 1 shows incorporation of C^{14}-labeled tyrosine and leucine into the pH 5 enzyme fraction of guinea-pig liver. Incorporation at zero time or in the absence of ATP is less than 5 per cent of that of the complete system. Enzyme preincubated for 10 minutes at 25° with 0.2 μg. of ribonuclease/ml is also inactive. Incorporated amino acid is released by ribonuclease and by dilute alkali, indicating binding to RNA.

TABLE 1

FRACTIONATION OF INCORPORATING AND ACTIVATING SYSTEMS

FRACTION	AMINO ACID INCORPORATION Total Activity in mμmoles* Tyrosine	Leucine	PYROPHOSPHATE EXCHANGE Total Activity in mμmoles* Tyrosine	Leucine
pH 5 enzyme	0.21	0.89	700.0	1,200.0
"Gel super"	0.01	0.05	200.0	420.0
"Gel eluate"	0.16	0	350.0	0
"Gel super" plus "gel eluate"	0.14	0.62	—	—
Heated "eluate"†	0.01	0	0	0
Heated "eluate" plus "gel super"	0.15	0.68	—	—
Heated "gel super" plus "eluate"	—	0.07	—	—
AS_{30-60} plus heated "eluate"	0.18	0	65.0	0

* The results are given for 1 ml. of pH 5 enzyme or its equivalent, so that the recovery of enzymatic activity in the various fractions can be calculated from the data. The spaces with dashes indicate "not measured."
† Heated at 80° for 10 minutes.

Fractionation of the crude pH 5 enzyme with calcium phosphate gel gives two fractions—the "gel eluate" and the "gel super" (Table 1). Neither fraction alone is active for leucine incorporation, but almost full activity is restored when the fractions are recombined. The values for pyrophosphate exchange (Table 1)

indicate that activating enzyme for leucine is present only in the "gel super" and not in the "gel eluate." The heat-stable component of the "gel eluate" is therefore not required for activation but is required in addition to activating enzyme for incorporation activity.

The "eluate fraction" alone incorporates tyrosine in good yield (Table 1). Tyrosine-activating enzyme is present in this fraction, however. The activating enzyme in the "eluate" is destroyed by heating, and, since tyrosine-activating enzyme is also present in the "gel super," "heated eluate" plus "gel super" restores incorporating activity for tyrosine (Table 1). Thus the tyrosine-incorporating system also consists of a heat-stable component and an activating enzyme fraction but differs from the leucine system in the adsorption of most of the tyrosine-activating enzyme by the gel. Tyrosine-activating enzyme of hog pancreas is also separated from other activating enzymes by this procedure.[6] The tyrosine-incorporating system of the "gel eluate" is purified twentyfold over the original pH 5 enzyme because of the separation of the activating enzyme by this step. Fractionation of the "gel super" with ammonium sulfate yields a tyrosine-activating enzyme free of leucine-activating enzyme, and this fraction will restore only tyrosine incorporation to the heated "eluate" (Table 1). Similarly, a leucine-activating fraction, free of tyrosine-activating enzyme, will restore only leucine incorporation (data not shown). These results provide, for the first time, direct evidence that specific amino acid-activating enzymes are required for the incorporation of specific amino acids but do not exclude the participation of other factors in the activating enzyme fractions. The occurrence of activating enzymes in the pH 5 fraction[7] and the correlation between activation and incorporation of tryptophan analogues[8] have previously been cited as evidence for the role of activating enzymes in amino acid incorporation.

Several considerations suggest that the "eluate" fraction (Table 1) contains RNA and that this is the "acceptor" for the activated amino acid. Thus the "eluate" contains 400–500 μg. of RNA/ml[9] and 500–600 μg. of protein.[10] This fraction is inactivated by ribonuclease, in contrast to the activating enzyme fraction (Table 2),

TABLE 2

RECOMBINATION OF ACTIVATING ENZYME WITH RNA

FRACTION	AMINO ACID INCORPORATED* Total Activity in mμmoles Tyrosine	Leucine
Ribonuclease-treated "gel super" plus "eluate"	—	0.55
Ribonuclease-treated "gel eluate" plus "super"	—	0.06
RNA from "gel eluate" plus "super"	0.12	0.62
RNA from microsomes plus "gel super"	0.01	0.03

* These results are comparable with those given in Table 1, although different enzyme preparations were used. Neither the "gel super" used for these experiments nor the RNA showed incorporation separately.

which is not. Finally, treatment of the "gel eluate" with phenol by a procedure patterned after the method of Kirby[11] yields RNA, which can substitute completely for the "eluate" (Table 2). RNA prepared by phenol treatment from the "eluate fraction" is, in fact, as active for incorporation as the original material. The activity shown in Table 2 represents the yield of RNA obtained by the extraction procedure rather than any inactivation. This yield ranges from 60 to 90 per cent

of the RNA present in the particular fraction. RNA prepared from guinea-pig liver microsomes by the same procedure is inactive (Table 2). Various other RNA preparations, such as those from virus and yeast, are also inactive. However, RNA from the pH 5 enzyme fraction of rat liver is fully active with activating enzymes from guinea-pig liver. RNA prepared by extraction with hot 2.0 *M* sodium chloride[12] also possesses high incorporation activity in this system.

Finally, it appears that specific RNA's are needed for the incorporation of individual amino acids. Under the usual conditions, there is a definite upper limit of incorporation for each amino acid. For leucine this is regularly four- to fivefold greater than for tyrosine (Table 1). For threonine, it is threefold greater than for tyrosine. Table 3 shows that incorporation of two amino acids—tyrosine and threonine—is additive. Furthermore, addition of non-radioactive threonine does not inhibit incorporation of radioactive tyrosine and vice versa. These experiments were done under conditions of maximal incorporation, so that available RNA is the limiting factor. Since each amino acid is incorporated independently, each must, apparently, be incorporated into a specific RNA. Preliminary fractionation studies appear to support this conclusion, since a "gel super" containing leucine-acceptor RNA, but not tyrosine-acceptor RNA, has been prepared. These experiments suggest that the isolation of specific RNA and the correlation of RNA structure with a biological activity are now possible.

TABLE 3

TYROSINE AND THREONINE COMPETITION

Additions	Amino Acid Incorporated* Total Activity in mμmoles
C^{14}-tyrosine	0.15
C^{14}-threonine	0.44
C^{14}-tyrosine plus C^{14}-threonine	0.56
C^{14}-tyrosine plus cold threonine	0.15
C^{14}-threonine plus cold tyrosine	0.46

* The enzyme fraction used was the "gel eluate" equivalent to 1 ml. of original pH 5 enzyme. The standard assay conditions were used. The non-radioactive (cold) amino acids were added at the level usually used in the assay.

Summary.—A soluble enzyme system which incorporates labeled amino acid into RNA, has been separated into two components. These are activating enzyme fraction and RNA acceptor. Both are needed for incorporation of amino acids. Activating enzyme fractions specific for tyrosine and leucine are required for the incorporation of the respective amino acid. The RNA acceptors appear also to be specific for each amino acid.

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*FLUORESCENCE STUDIES OF COENZYME BINDING TO DEHYDROGENASES**

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The mechanism of binding of pyridine coenzymes to dehydrogenases has been studied principally by two techniques. One of these is the ultracentrifugal method,[1] and the other, which has been used more extensively, is the spectrophotometric measurement of characteristic bands of the enzyme-coenzyme complex. Theorell and Bonnichsen[2] reported a shift in the spectrum of reduced diphosphopyridine nucleotide (DPNH) in the presence of horse liver alcohol dehydrogenase, and Boyer and Theorell[3] have also reported a shift in the fluorescence spectrum of DPNH upon the addition of liver alcohol dehydrogenase.

Recently, studies in coenzyme binding to dehydrogenases have been facilitated by the preparation of analogues of DPN in which the nicotinamide moiety is replaced by other pyridine derivatives.[4] While Chance and Nielands[5] could demonstrate a spectral shift of DPNH with beef heart lactic dehydrogenase (LDH) only at low temperatures and with a "sensitive" spectrophotometer, Kaplan, Ciotti, and Stolzenbach[6] have demonstrated a shift of the reduced acetyl pyridine analogue of DPN (APDPNH) at room temperature. This report deals with the fluorescence changes associated with the addition of APDPNH to crystalline beef heart LDH.

Methods and Materials.—The beef heart and rabbit muscle lactic dehydrogenase were obtained by the respective methods of Straub[7] and Kornberg.[8] Horse liver ADH was a crystalline product prepared by the procedure of Bonnichsen and Brink.[9] The coenzyme analogue was prepared as described by Kaplan and Ciotti.[10]

Sodium *p*-chloromercuribenzoate (PCMB) was obtained from the Sigma Chemical Company and purified as described by Boyer.[11]

The fluorescence spectra were studied with the Aminco-Bowman spectrophotofluorometer manufactured by the American Instrument Company. This fluorometer has been described by Bowman *et al.*[12]

Results.—When a solution of APDPNH is excited by ultraviolet light having a wave length of 365 mμ, a fluorescence with its maximum at 480 mμ is emitted. Upon the addition of small quantities of crystalline beef heart LDH, the maximum