thiohydantoin which was identified chromatographically as tyrosine PTH. In all, the material on the paper strip was submitted to eight successive applications of the PTC procedure; the results obtained are summarized in Table I. The phenylthiohydantoins were subjected to paper chromatography, each in the two-solvent systems described above, and identified by comparison of their $R_f$'s with those of control phenylthiohydantoins which were run concurrently. Furthermore, the PTH derivatives of tyrosine, methionine, glutamic acid and phenylalanine were chromatographically identified as the corresponding amino acids, after hydrolysis in sealed evacuated tubes with 6 $N$ HCl at 150° for 16 hours. From these results it is concluded that α-corticotropin is composed of a single polypeptide chain, having the N-terminal[6] heptapeptide sequence ser.tyr.ser.met.glu.his.phe....

TABLE I

STEPWISE DEGRADATION OF α-CORTICOTROPIN

| Step no. | Phenylthiohydantoin Yield,[a] $M$/4500 g. | Phenylthiohydantoin Residue[b] |
|---|---|---|
| 1 | 0.85 | Serine |
| 2 | .83 | Tyrosine |
| 3 | .80 | Serine |
| 4 | .85 | Methionine |
| 5 | .81 | Glutamic acid |
| 6 | .58 | Histidine |
| 7 | .62 | Phenylalanine |
| 8 | < .05 | Unidentified |

[a] Estimated from optical density readings at 270 mμ, assuming $\epsilon$ = 16,000. [b] Identified by paper chromatography according to Sjöquist.[5]

The amino acid sequence from the carboxyl end of the peptide was investigated with the aid of carboxypeptidase by the procedure previously described.[7] α-Corticotropin trichloracetate (5.0 mg.) was incubated with diisopropylfluorophosphate-treated carboxypeptidase (0.25 mg.) in a 5.0 ml. solution at $p$H 8.5 and a temperature of 40°; aliquots of the digest (1.0 ml.) were removed at suitable time intervals and allowed to react with dinitrofluorobenzene at $p$H 9.0 and 40° for the separation and quantitative estimation[8] of the free amino acids released during the enzymatic reaction. The results indicated that phenylalanine, glutamic acid, and leucine are released successively by the stepwise degradation of the C-terminal sequence[6] . . . .leu.glu.phe in the α-corticotropin molecule. Furthermore, if it is assumed that one mole of C-terminal amino acid is released per mole of α-corticotropin, a value of 4,500 (±200) may be calculated for its equivalent weight which is in excellent agreement with the minimum molecular weight based on amino acid analysis.[9]

The fact that no further significant digestion took place after equivalent stoichiometric amounts of phenylalanine, glutamic acid and leucine had been released in the course of the carboxypeptidase reaction suggested that the fourth amino acid along the chain is one which, failing to conform to the specificity requirements of the enzyme, resists digestion; for example, proline. This prediction was confirmed when the tetrapeptide pro.leu.glu.phe,[6] and the tripeptide pro.leu.glu ($R_f$'s 0.83 and 0.75, respectively, on Whatman No. 1 paper using butanol/acetic acid/$H_2O$, 4:1:5 as the solvent system) were isolated and characterized from a pepsin digest (enzyme/substrate mole ratio 1:250, $p$H 2.0, 40°, 8 hours) of α-corticotropin. Since only one residue of leucine is present in the molecule, the tetrapeptide pro.leu.glu.phe is shown to be the C-terminal sequence in α-corticotropin.

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(6) Ser.tyr has been identified as the N-terminal dipeptide (W. A. Landmann, *et al.*, THIS JOURNAL, **75**, 4370 (1953); S. W. Fox, *et al.*, *ibid.*, **76**, 1154 (1954)), and pro.leu.glu.phe as the C-terminal tetrapeptide (W. F. White, *ibid.*, **75**, 4877 (1953)) sequences in Corticotropin A isolated from *hog* pituitary glands.

(7) J. I. Harris in D. Glick, "Methods of Biochemical Analysis," Vol. II, Interscience Publishers, New York, in press; J. I. Harris, C. H. Li, P. Condliffe and N. G. Pon, *J. Biol. Chem.*, **209**, 133 (1954).

(8) A. L. Levy, *Nature*, in press; A. L. Levy and D. Chung, to be published.

## ENZYMATIC SYNTHESES OF PYRIMIDINE AND PURINE NUCLEOTIDES.[1] III. FORMATION OF NUCLEOSIDE DIPHOSPHATES AND TRIPHOSPHATES

*Sir:*

Extracts of yeast and liver which convert orotic acid to uridine-5′-phosphate (U5P) also bring about the synthesis of uridine diphosphate (UDP) and uridine triphosphate (UTP).[2] While several pathways are known for the formation of UTP,[3] no mechanism has been elucidated for its synthesis from U5P. We have now obtained evidence with the use of a partially purified enzyme from yeast for the conversion of U5P to UTP by myokinase-like reactions (equation (1), and illustrated by equations (2, 3)). Unlike muscle myokinase,[4] which acts on adenosine nucleotides only, this yeast enzyme effects a transphosphorylation between uridine and adenosine nucleotides.

(1) Nucleoside-P + *nucleoside*-P-P-P ⇌ nucleoside-P-P + *nucleoside*-P-P

(2) U5P + adenosine triphosphate (ATP) ⇌ UDP + adenosine diphosphate (ADP)

(3) U5P + UTP ⇌ 2 UDP

Like U5P, adenosine-5′-phosphate (A5P) is phosphorylated by the same enzyme preparation by analogous reactions (equations (4, 5)).

(4) A5P + UTP ⇌ ADP + UDP

(5) A5P + ATP ⇌ 2ADP

Preliminary spectrophotometric evidence for the formation of nucleoside diphosphates (equations (2–5)) was obtained by the use of the phosphopy-

(1) This investigation was supported by research grants from the National Institutes of Health, Public Health Service.

(2) A. Kornberg, I. Lieberman and E. S. Simms, THIS JOURNAL, **76**, 2027 (1954); I. Lieberman, A. Kornberg and E. S. Simms, *ibid.*, **76**, 2844 (1954).

(3) A. Kornberg, "Phosphorus Metabolism," Vol. I, edited by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1951; A. Munch-Petersen, H. M. Kalckar, E. Cutolo and E. E. B. Smith, *Nature*, **172**, 1036 (1953); P. Berg and W. K. Joklik, *ibid.*, **172**, 1008 (1953).

(4) S. P. Colowick and H. M. Kalckar, *J. Biol. Chem.*, **148**, 117 (1943); H. M. Kalckar, *ibid.*, **148**, 127 (1943).

ruvate-pyruvate phosphokinase system coupled to the DPNH-lactic dehydrogenase system.[5,6] More direct evidence for equations (1–5) was obtained by ion-exchange chromatographic analysis of the reaction products in 2 experiments. In the reaction of UTP and A5P ($C^{14}$-labeled) (Table I),[7] roughly equal concentrations of the mono-, di- and triphosphates of uridine and adenosine were obtained. The identity and quantity of each component was determined by spectrophotometric measurements and, where indicated, by radioactivity measurements and enzymatic assays. No reaction occurred when either UTP or A5P was omitted from the incubation mixture.

TABLE I: TRANSPHOSPHORYLATION BETWEEN ADENINE AND URACIL NUCLEOTIDES

The reaction mixture (2.05 ml.) contained 0.2 ml. of glycylglycine buffer (0.5 *M*, *p*H 7.5), 0.1 ml. of $MgCl_2$ (0.1 *M*), 0.5 ml. of 2-$C^{14}$-A5P (0.002 *M*, 82,000 c.p.m./μmole), 0.25 ml. of UTP (0.004 *M*), and 1.0 ml. of the heated enzyme preparation (fraction I heated in a boiling water-bath for 3 minutes at *p*H 1.5; 0.57 mg. of protein). After incubation at 36° for 1 hour, the reaction mixture was heated in a boiling water-bath for 3 minutes.

| | 0 min. μmoles | 60 min. μmoles | Δ μmoles | Δ Total c.p.m. |
|---|---|---|---|---|
| A5P[a,b] | 0.89 | 0.30 | −0.59 | −45,260 |
| UTP[c] | .83 | .32 | − .51 | |
| ADP[d] | .00 | .33 | + .33 | +26,800 |
| ATP[e] | .00 | .28 | + .28 | +23,100 |
| U5P[f] | .00 | .31 | + .31 | |
| UDP[g] | .00 | .35 | + .35 | |

[a] Anion-exchange chromatography of the reaction mixture yielded A5P and UTP free from each other and the other nucleotides; ADP and U5P were eluted together; ATP and UDP were separated except in a few fractions. [b] Estimated spectrophotometrically at 260 mμ and by radioactivity measurements. [c] Estimated spectrophotometrically at 260 mμ. [d] Estimated by radioactive measurements and with pyruvate phosphokinase (0.29 μ mole found). [e] Estimated by radioactivity measurements. Fractions assayed with hexokinase and glucose-6-phosphate dehydrogenase (A. Kornberg, *J. Biol. Chem.*, **182**, 779 (1950)), gave results in agreement with the radioactivity measurements. [f] Estimated spectrophotometrically at 260 mμ correcting for the ADP present. [g] Estimated spectrophotometrically at 260 mμ correcting for the ATP present. Fractions assayed with pyruvate phosphokinase gave results in agreement with the spectrophotometric data.

A second experiment demonstrates the stoichiometry of the reaction between UTP and U5P ($C^{14}$-labeled) (Table II).[7] In the absence of either UTP or U5P, no UDP was formed. UDP, isolated chromatographically, was identified by its absorption spectrum (max. at 262 mμ, λ280/λ260 = 0.37 in *N* HCl) and by the molar ratios of uracil:acid-labile phosphate:total phosphate:phosphate acceptor (in the phosphopyruvate-pyruvate phosphokinase system) of 1.00:0.97:1.94:0.94.

The enzyme activity responsible for reactions (2–5) is relatively stable to heating at acid *p*H (47% loss after 3 min. at 100° at *p*H 1.3). Thus it resembles the myokinase (adenylate kinase) of yeast[5] in this respect; its behavior on fractionation is also similar. While these findings suggest that a single enzyme is responsible for the mixed myokinase reaction described in equations (1–5), further work is necessary to establish this point.[8] The absence of the nucleoside diphophoskinase of Berg and Joklik[9] in our preparation was demonstrated spectrophotometrically by the failure of ATP to react with inosine diphosphate.

These results and reports of natural occurrence of nucleoside di- and triphosphates,[9] make it likely that enzymes with comparable functions to this yeast enzyme are widespread in nature.[10]

(5) A. Kornberg and W. Pricer, Jr., *J. Biol. Chem.*, **193**, 481 (1951).

(6) With ATP and various nucleoside monophosphates, this assay system was used to study the specificity of the enzyme. Phosphate transfer from ATP was measured by the appearance of pyruvate. In addition to U5P and A5P, desoxyadenosine-5′-phosphate and guanosine-5′-phosphate were active. Apparently inactive were the 5′-substituted phosphate esters of inosine, desoxycytidine, desoxyguanosine and nicotinamide riboside, and adenosine-3′-phosphate.

(7) The substrates were purified by anion-exchange chromatography.

TABLE II

STOICHIOMETRY OF UDP SYNTHESIS FROM U5P AND UTP

The reaction mixture (2.0 ml.) contained 0.2 ml. of glycylglycine buffer (0.5 *M*, *p*H 7.5), 0.1 ml. of $MgCl_2$ (0.1 *M*), 0.2 ml. of 2-$C^{14}$-U5P (0.01 *M*, 17,000 c.p.m./μ mole), 0.45 ml. of UTP (0.004 *M*), and 0.8 ml. of the enzyme preparation (fraction III, containing 0.26 mg. of protein). After incubation at 36° for 45 minutes, the reaction mixture was heated in a boiling water-bath for 3 minutes.

| | 0 min. μmoles | 45 min. μmoles | Δ μmoles | Δ c.p.m. |
|---|---|---|---|---|
| U5P[a] | 2.20 | 1.81 | −0.39 | −9270 |
| UTP[a] | 1.64 | 1.27 | −0.37 | +5220[c] |
| UDP[a,b] | 0.00 | 0.82 | +0.82 | +5330[c] |

[a] Estimated spectrophotometrically at 260 mμ after chromatography on Dowex-1 anion-exchange resin. [b] Estimated with pyruvate phosphokinase. [c] The specific activity of the UTP is relatively high and that of the UDP, low.

(8) NOTE ADDED IN PROOF.—Further studies have revealed that heating of the enzyme preparation at neutral *p*H (100°, 3 min.) results in a recovery of 14.6% of the adenylate kinase activity (equation (5)) but less than a 1% recovery of the uridylate kinase activity (equations (2) and (3)). This evidence indicates the presence of two distinct enzyme activities in our preparation.

(9) R. Bergkvist and A. Deutsch, *Acta Chem. Scand.*, **7**, 1307 (1953); H. Schmitz, V. R. Potter, R. B. Hurlbert and D. M. White, *Cancer Research*, **14**, 66 (1954).

(10) NOTE ADDED IN PROOF.—Dr. A. Munch-Petersen and Dr. D. R. Sanadi have observed reactions similar to those described in this report (personal communications) in yeast and animal tissues, respectively.

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## THE AMALGAM PARTITION METHOD FOR THE DETERMINATION OF IONIC FREE ENERGIES IN NON-AQUEOUS SOLUTIONS[1]

*Sir:*

The standard free energies of formation of ions in solution, although important to an understanding of the properties of electrolytic solutions, are practically unknown except for aqueous solutions. The only widely applicable methods previously available for obtaining such data are measurements of the potentials of reversible e.m.f. cells, of the solubility equilibria of pure electrolytes of known free energy, and of the partition equilibria between immiscible aqueous and non-aqueous electrolyte solutions.[2] A new method is described here which leads to the relative free energies of formation of metallic ions in suitable non-aqueous solvents.

This method involves equilibria of the type

$$M^+ + N(Hg) = M(Hg) + N^+ \qquad (1)$$

(1) This work was supported by the Atomic Energy Commission.

(2) H. L. Friedman and G. R. Haugen, THIS JOURNAL, **76**, 2060 (1954).