mediates in biological peptide bond synthesis is lacking. We report here enzyme-catalyzed synthesis of carnosine (β-alanyl-L-histidine) from β-alanyl adenylate and histidine. Carnosine synthesis from β-alanine and L-histidine was observed in the presence of ATP, Mg^{++} and soluble enzyme preparations obtained from chicken and rabbit skeletal muscle,[3] by extraction of the muscle with

TABLE I

Exp.	Reaction mixtures[a]	C^{14}-Carnosine (c.p.m.)[e]
1	C^{14}-β-Alanine + L-histidine + ATP[b]	13,100
2	β-Alanine + ATP + C^{14}-DL-histidine[b]	12,900
3	β-Alanyl-adenylate[c] + C^{14}-DL-histidine	15,800
4	β-Alanyl-adenylate + C^{14}-DL-histidine[d]	2,750

[a] Composition of reaction mixtures; Exp. 1. β-alanine-1-C^{14} (250,000 c.p.m., 0.08 micromole), L-histidine (3 micromoles), ATP (2.5 micromoles), $MgCl_2$ (3 micromoles), KCl (100 micromoles), sodium phosphate buffer (67 micromoles) and enzyme (10 mg.); *p*H 7.4 final volume, 1 ml.; incubated for 20 min. at 37°. Exp. 2–4. β-alanine (3 micromoles), ATP (2.5 micromoles), β-alanyl adenylate (3.8 micromoles), DL-histidine-2-C^{14} (0.8 micromole), 416,000 c.p.m.); no $MgCl_2$ or KCl was added in exp. 3 and 4; other conditions were as in exp. 1. [b] No carnosine was formed when any one of these components, $MgCl_2$, or enzyme was omitted, or when guanosine triphosphate, cytidine triphosphate, uridine triphosphate, or inosine triphosphate was used at equivalent concentrations in place of ATP. Substitution of equimolar concentrations of $CoCl_2$ or $MnCl_2$ for $MgCl_2$ gave less than 25% of the reported activity. [c] No carnosine was formed when β-alanyl-adenylate was hydrolyzed prior to use. [d] Enzyme was omitted or heated at 100° for 10 min. prior to use. [e] Separated by ascending paper chromatography on Whatman No. 3 paper and a solvent consisting of ethanol, acetic acid, H_2O (75:5:20); other solvents, *e.g.*, butanol, formic acid, H_2O (75:15:10), phenol, H_2O (80:20; saturated with HCl), gave similar results.

a solution containing 0.65 *M* NaCl and 0.04 *M* $KHCO_3$, and then fractionation with ethanol at −5 to −10°. Formation of C^{14}-carnosine from C^{14}-β-alanine or C^{14}-histidine was demonstrated by paper chromatography in several solvents; the C^{14}-carnosine was eluted and hydrolyzed with hydrochloric acid or carnosinase[4] to yield the C^{14}-precursors. No carnosine was formed when histidine, β-alanine, Mg^{++}, ATP, or enzyme were separately omitted. Substitution of equivalent quantities of D-histidine[5] for L-histidine gave less than 5% of the radioactivity in the carnosine area, while a compound with the properties of anserine (β-alanyl-1-methyl-L-histidine) was formed when histidine was replaced by 1-methylhistidine. Addition of KCl was required for optimum activity with certain enzyme fractions; added coenzyme A (0.002 *M*) did not affect the rate of synthesis. Addition of pyrophosphatase (50 micrograms per ml.)[6] increased synthesis by about 25%.

When β-alanyl adenylate, synthesized as previously described,[7] was added to the reaction mixture in place of ATP, Mg^{++}, KCl, and β-alanine, considerable synthesis of carnosine was observed (Table I). Under these conditions some radioactivity appeared in the carnosine area in the absence of enzyme. Enzymatic formation of carnosine was not observed in experiments with β-alanyl phosphate. The results suggest that carnosine synthesis involves activation of β-alanine to yield β-alanyl adenylate, and reaction of this anhydride with histidine to form carnosine. Mg^{++} ions appear to be required only for activation. In analogy with tryptophanyl adenylate,[1] β-alanyl adenylate may be tightly bound to enzyme, and therefore studies with relatively large amounts of purified enzyme may be required for isolation of enzymatically-formed β-alanyl adenylate.[8]

(3) While this work was in progress, T. Winnick and R. E. Winnick (Abstracts, 4th International Congress of Biochemistry, Vienna, 1958) reported synthesis of carnosine from β-alanine and histidine by chick muscle preparations.

(4) H. T. Hanson and E. L. Smith, *J. Biol. Chem.*, **179**, 789 (1949).

(5) Generously donated by Dr. Jesse P. Greenstein.

(6) L. A. Heppel, in "Methods in Enzymology" (Colowick and Kaplan, eds.) **2**, 570 (1955).

(7) P. Castelfranco, K. Moldave and A. Meister, THIS JOURNAL, **80**, 2335 (1958); "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.), Wash. Acad. Sci., 115 (1958).

(8) We wish to thank the National Heart Institute (National Institutes of Health) of the Public Health Service and the National Science Foundation for generous support of this research; abbreviation ATP, adenosine triphosphate.

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RECEIVED JANUARY 22, 1959

AMPEROMETRIC DETERMINATION OF FLUORIDE AT A ROTATED ALUMINUM ELECTRODE

Sir:

From 99.994% pure aluminum wire (1/16 inch diameter) a rotated electrode (600 r.p.m.) has been constructed of about 0.15 $cm.^2$ surface area. After each set of measurements it was "cleaned" in a 0.01 *M* ethylenediaminetetraacetate buffer of *p*H 3. All experiments were carried out at 25°. In nitric acid solutions of *p*H 2 or less and acetate buffers of *p*H 3 to 5.5 the electrode is highly polarized. Anodic depolarization occurs at about +0.6 volt and cathodic depolarization at −1.4 volt (*vs.* S.C.E.). Perchlorates and halides exert an anodically depolarizing effect at about −0.6 volt, but they do not depolarize cathodically.

In acid medium the zero current potential is ill-defined and extremely poorly reproducible. Fluoride in acid medium depolarizes the electrode anodically, it causes the potential to become extremely negative. Current–potential curves in the presence of fluoride are of a composite nature. In the absence of oxygen anodic limiting currents are found at −0.75 volt which are proportional to the fluoride concentration in a range between 10^{-5} and 3×10^{-4} *M*. In the amperometric determination of fluoride in an oxygen-free acetate buffer of *p*H 3.5 use is made of the "standard addition" method which makes the accuracy independent of the reproducibility of the surface conditions of the electrode. Alkali perchlorates, nitrates, chlorides, sulfates and 0.002 *M* calcium do not interfere. Phosphate decreases the proportionality factor between limiting current and concentration but *does not interfere*. The results of the amperometric method have been tested in Minneapolis city water and eight natural waters and good agreement with results obtained by classical methods has been obtained. Baker and Morrison[1] determined microgram quantities of fluoride by spontaneous elec-

(1) B. B. Baker and J. D. Morrison, *Anal. Chem.*, **27**, 1306 (1955).

trolysis in a cell provided with an aluminum electrode and a platinum spiral, but their results could not be substantiated.

We have successfully applied the rotation aluminum electrode to the amperometric titration of fluoride in aqueous buffers with aluminum nitrate; no good end-point was observed with thorium nitrate as reagent. Hydroxyl ions are the only other ions which anodically depolarize the electrode. They yield anodic limiting currents proportional to concentration. Use of this can be made in the amperometric titration of acids with a strong base. At a *p*H greater than 8 the electrode acts as a *p*OH (*p*H) electrode, but not in acid medium. In acid medium it acts like a *p*F electrode, the potential being somewhat dependent on *p*H in the presence of fluoride. Details concerning this interesting electrode will be reported elsewhere.

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RECEIVED FEBRUARY 2, 1959

INHIBITION OF PYRIDOXAL PHOSPHATE-DEPENDENT ENZYMES BY THE SULFATE ESTERS OF ESTRADIOL, ESTRONE AND DIETHYLSTILBESTROL[1]

Sir:

Systematic studies of kynurenine transaminase in this laboratory have revealed that it is inhibited by extremely low concentrations of estradiol disulfate and diethylstilbestrol disulfate(Table I). Estrone sulfate and pregnanediol glucuronide also inhibited but only at considerably higher levels. Several bile acids inhibited slightly at high concentrations (10^{-3} to 10^{-4} *M*), probably by denaturation. Unconjugated steroids and related compounds, *e.g.*, estradiol, estrone, diethylstilbestrol, progesterone, pregnanediol, methyl bisdehydrodoisynolic acid and methallenestril were without effect even at saturation levels.

The degree of inhibition varied with the concentration of pyridoxal phosphate, indicating that the estrogen sulfates may compete with the coenzyme for the apotransaminase. Reversibility was shown also by complete disappearance of the inhibitory effect during dialysis. The possibility, suggested here, that other pyridoxal phosphate-dependent enzymes may be inhibited led to tests of the effects of the estrogen sulfates on the activity of muscle phosphorylase (a crystalline preparation) and to the discovery that they inhibit this enzyme also and at very low levels (Table I).

Kynurenine transaminase was purified and assayed as described earlier.[2] Twice crystallized muscle phosphorylase *a* was dissolved in cysteine-glycerophosphate buffer, *p*H 6.8. Aliquots were preincubated at 37° for 30 minutes with the various inhibitors or an equal volume of water, then assayed for activity by the method of Illingworth and Cori.[3]

TABLE I

INHIBITION OF KYNURENINE TRANSAMINASE AND MUSCLE PHOSPHORYLASE BY STEROID CONJUGATES

Inhibitor	Inhibitor concn., *M*	Per cent. inhibition: Kynurenine transaminase	Per cent. inhibition: Muscle phosphorylase *a*
Estradiol disulfate	2.5×10^{-5}	97	48
	5×10^{-6}	50	19
	5×10^{-7}	9	..
Diethylstilbestrol disulfate	2.5×10^{-5}	99	76
	5×10^{-6}	60	28
	5×10^{-7}	11	..
Estrone sulfate	1×10^{-4}	23	15
	2.5×10^{-5}	9	2
	5×10^{-6}	0	0
Pregnanediol glucuronide	1×10^{-4}	7	..
	2.5×10^{-5}	0	..

Muscle phosphorylase contains tightly-bound pyridoxal phosphate which can be removed to yield preparations that are enzymatically active only in the presence of added pyridoxal phosphate.[4] In the present studies, the reactivation of such preparations with pyridoxal phosphate was inhibited by low concentrations of the estrogen sulfate. The degree of inhibition again was dependent on the concentration of added pyridoxal phosphate.

We believe this to be a unique case of a highly-sensitive *in vitro* estrogen–enzyme interaction in which both diethylstilbestrol and the natural estrogens can participate. From a speculative viewpoint, many of the *in vivo* effects of the estrogens correspond rather well to what we might expect from the demonstrated effects on pyridoxal phosphate-dependent enzymes. As an example, the increased storage of glycogen and protein that occurs in some species under the influence of the estrogens may be regulated through the inhibition of phosphorylase and transaminase enzymes. Experiments designed to test further the relationship of these *in vitro* effects to the physiological actions of the steroid hormones are in progress.

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STEREOCHEMISTRY OF THE DECOMPOSITION OF OPTICALLY ACTIVE N-NITROSO AND N-AMINO α,α'-DIMETHYLIDIBENZYLAMINE

Sir:

Recently it has been shown that the reduction of *cis* or *trans* N-nitroso-2,6-diphenylpiperidine[1] and oxidation of the corresponding N-amino compounds[2] proceeded to give either the *cis* or *trans* diphenylcyclopentane and some olefin. The cyclic compounds were formed with retention of configuration. In order to determine whether the same retention of configuration was operative in linear homologs, optically pure N-nitroso-α,α'-

(1) This work was supported by a grant (A-2294) from the National Institutes of Arthritis and Metabolic Diseases, National Institutes of Health.

(2) M. Mason, *J. Biol. Chem.*, **227**, 61 (1957).

(3) B. Illingworth, and G. T. Cori, "Biochemical Preparations," Vol. 3, John Wiley and Sons, New York, N. Y., 1953, p. 1.

(4) C. F. Cori and B. Illingworth, *Proc. Nat. Acad. Sci.*, **43**, 547 (1957).

(1) C. G. Overberger, J. G. Lombardino and R. G. Hiskey, THIS JOURNAL, **80**, 3009 (1958).

(2) C. G. Overberger, J. G. Lombardino and R. G. Hiskey, *ibid.*, **79**, 6430 (1957).