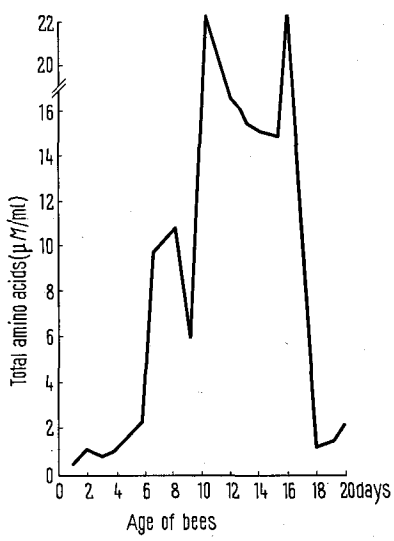
Approximately 1 ml of fluid or hemolymph from each age group of honey bees was collected in a sterile glass test tube and frozen prior to analysis. Eggs were placed in a tube and macerated. Hemolymph from larvae was obtained by gently puncturing the insects with a sterile hypodermic needle. The fluid which exuded from the wound was drawn into a capillary tube and then expelled into a test tube. Fluid from pupae and hemolymph from adults were best obtained by severing the head with a scalpel and drawing the liquid which exuded from the thorax into a capillary tube.

Each sample was lyophilized, flushed twice with nitrogen, and hydrolyzed with 6 *N* HCl in sealed tubes. After the tubes were heated for 24 h at 108 °C, the hydrolysate was removed, filtered, and dried. Distilled water was added twice during the evaporation procedure to remove excess HCl. After the final evaporation, the volume of the residue was adjusted to 25 ml with citrate buffer, ph 2.2. The sample was then examined with a Beckman Model 121 amino acid analyzer[10].

The total amino acid levels were lowest in eggs of all ages, in hemolymph from 5- and 6-day-old larvae, in fluid from 18- and 19-day-old pupae, and in the hemolymph of emerging adult bees (Figure). Hemolymph from 10-day-old larvae and fluid from 16-day-old pupae contained the greatest amounts of amino acids. Amino acid levels from day 10 larvae through day 16 pupae were consistently high. Obviously, these compounds were involved in protein synthesis during pupation which occurred at day 11 and in preparation for emergence of the adult at day 20. The sharp decreases in amino acids that occurred at days 9, 11, 17, and 18 reflected the morphological events of pupation and emergence which were occurring.



Total amino acids in developing worker honey bees.

The Table shows total amino acids present in developing worker honey bees. LUE and DIXON[6] found proline, glutamic acid, serine, glycine, and alanine nonessential to bees. The highest values we found were those for glutamic acid, aspartic acid, and proline. CHEN and HADORN[11] reported a high concentration of proline in the blood of *Ephestia* spp. but could offer no explanation for this. Proline, aspartic acid, and glutamic acid were interrelated in the silkworm[12], and this appeared to be true for the honey bee also. Those amino acids present in the lowest concentrations were 1/2 cystine, histidine, methionine, and phenylalanine. MILES[13] found low concentrations of free cysteine-cystine in the hemolymph of *Eumecopus punctiventris* Stål. All amino acid levels increased from day 18 to day 20 (emerging bees).

It has rarely been possible to associate changes in individual amino acids with specific developmental processes[14] and no such correlation could be made in this study. However, we anticipate that correlations will be made in the future with improved methodology and better understanding of the developmental processes of insects[15].

Zusammenfassung. Glutaminsäure, Aspartinsäure und Prolin wurden in Eihomogenaten und Haemolymphe der Honigbiene (*Apis mellifera* L.) gefunden.

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[10] Mention of a proprietary product in this paper does not constitute an endorsement of this product by the USDA.

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Redox Enzyme Models from Polymerized Aminoacyladenylates

The difficulties of ascertaining enzyme reaction mechanisms by direct means of investigation has often led to model system studies[1,2]. Our studies of mechanism in the flavin mono-nucleotide (FMN) reduced nicotinamide ademine dinucleotide (NADH) model system[3,4] for reduced pyridine dinucleotide flavoenzyme dehydrogenases produced an initial reaction rate which was 10^{-6} the rate of the oxidation of NADH by the NADH dehydrogenase[5] from bovine heart mitochondria. WANG[6] had proposed that the enhancement of reaction rate for heme-oxygen interaction in hemoglobin was the result of the prosthetic group residing in a protein region of low dielectric strength. Studies on heme-catalyzed oxidations following the incorporation of heme into

polylysine and polystyrene gave experimental support to this thesis[7]. Perutz calculates the dielectric strength of the heme in its globin environment to be around 2–3.

In a study of the role of heteropolyamino acids, including protein, in enzymic catalysis, we chose to find a redox enzyme model of mixed amino acids which had not been the result of evolutionary specialization. KRAMPITZ and FOX[8] had reported that the aqueous polymerization of aminoacyl adenylates would form polypeptides with molecular weights of up to 150,000 if a polyamino acid core was used to initiate the polymerization. A similar system is now reported from Katchalsky's laboratory[9]. It has been recently reported that heme could be incorporated in such polymers[10], although no details were given.

Our procedure for synthesis of polyamino acids is very similar to one used by NAKASHIMA et al.[11]. The reaction employs 2.59 g of an equimolar mixture of 20 amino acids, 28.8 ml of H_2O, 93.6 ml of pyridine, 2.25 ml of 8 *N* HCl, 6.57 g of adenylic acid, and 0.25 g of formylmethionine. This is stirred in a dry ice-acetone bath in a hood. To this is added 91.8 g of dicyclohexylcarbodiimide (DCCD) in 105 ml of pyridine. This whole mixture is stirred at −20 °C for 3½ h. The dicyclohexylurea is then removed by vacuum filtration. The filtrate is separated in a separatory funnel; the bottom layer is saved. The aminoacyl adenylates in the bottom layer are next precipitated by 150 ml of dry ice cold acetone, yielding a gummy gel. This is washed with cold acetone-ethanol (6:4) and then ether. The polymerization to polyamino acids (called adenylate proteinoid here) is conducted as soon after this as possible in 4 g of sodium bicarbonate/25 ml of water, pH 9.6, for 1 h at room temperatures. The material is then dialyzed against water for one to two days and lyophilized. Yields of 50% calculated on starting amino acids are obtained.

Conducting the polymerization in the presence of hemin, chlorophyll, or flavin mononucleotide produced intensely red, green, or yellow colored products even after extensive dialysis. The presence of flavin mononucleotide in the reaction mixture caused a 50% reduction in the incorporation of basic residues, threonine, and phenylalanine (Table). The neutral and acidic residue contents were the same in the presence or absence of flavin mononucleotide in the reaction mixture and are the same as those reported by NAKASHIMA et al.[11]. The phosphate group on the FMN is no doubt the cause of the modification as basic residues were largely affected.

Of many techniques attempted for determination of molecular weight, Sephadex G-25 and G-15 chromatography appears to bracket the weight, at about 2.500, although quite anomalous behavior is noted by excessive retention times on G-50 and G-75. The elution profiles were of similar shape and width as those obtained for purified protein and peptide markers. These are interpreted as indicating a polymer of limited heterogeneity in molecular weight in the absence of core compound as also reported independently[9], and as has been reported also for thermal proteinoids[12].

The optical properties of the material obtained by polymerization in the presence of FMN are very similar to those of free FMN. Both absorption and fluorescence measurements indicate ¼ FMN/2,500 molecular weight. Sodium dithionite reduction occurs, but it is uncomplicated by the reduced flavin disproportionation reactions (see Figure 1). This result is similar to the observation for the NADH-FMN system, reported several years ago[4], in which the presence of thermally prepared proteinoid obliterated the free radical paramagnetic resonance signal. Optical rotatory dispersion gives a modified spectrum (Figure 2), however, when compared to any of the plausible flavin species. This result is tentatively indicative of a modified flavin environment. Comparison with the spectra of SIMPSON and VALLEE[13] shows a lack of correspondence with FMN, AMP, or FAD.

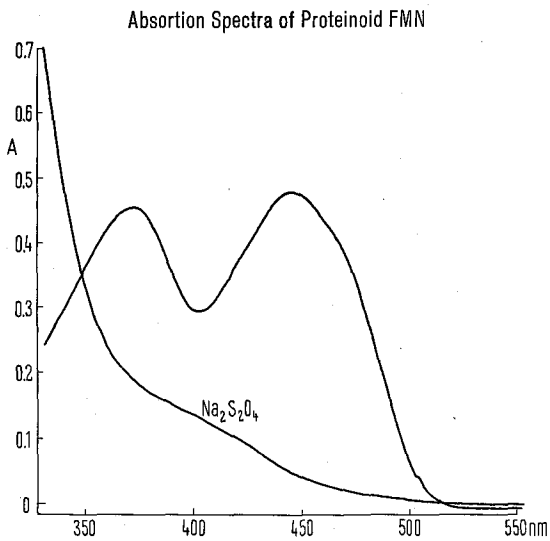

Fig. 1. Optical absorption spectrum of proteinoid from amino acyl adenylates polymerized with flavin mononucleotide in the oxidized state and following sodium dithionite reduction. 4 mg/ml proteinoid concentration.

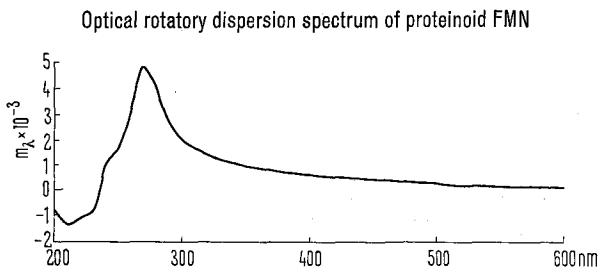

Fig. 2. Optical rotatory dispersion of proteinoid polymerized with flavin mononucleotide. 0.4 mg/ml concentration corresponding to 4.1×10^{-5} *M* FMN was used.

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Amino acid composition of amino acid adenylates polymerized to proteinoid in the presence and absence of flavin monoucleotide

Amino acids	Proteinoid — FMN	Proteinoid + FMN	Proteinoid + FMN / Proteinoid — FMN
Lysine	11.4 μmoles	7.4 μmoles	0.65
Histidine	6.8	4.0	0.59
Arginine	12.4	6.8	0.55
Threonine	4.3	2.5	0.58
Phenylalanine	4.4	2.8	0.64
Others	no significant change		

Analyses were performed on 0.4 mg aliquots on the Spinco 120B using the modification of HUBBARD[18].

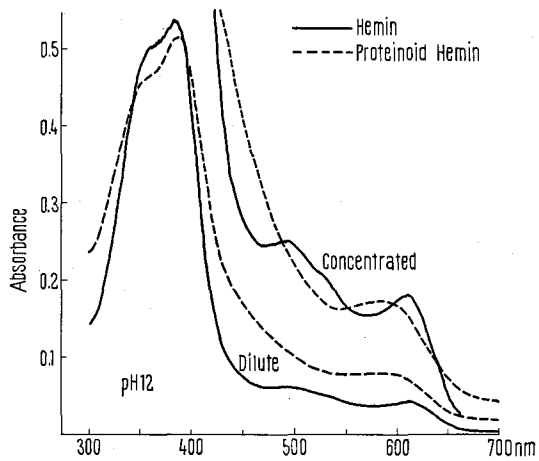

Fig. 3. Optical absorption spectra of proteinoid polymerized in presence of hemin compared to free hemin spectra.

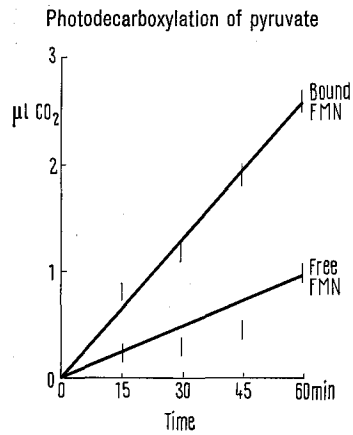

Fig. 4. Kinetics of photo-decarboxylation of pyruvate measured by Warburg manometry. 5 ml volume contained 4 mg/ml flavoproteinoid or 445 nm absorbance equivalent free FMN, 0.1 *M* pyruvate, and 0.01 *M* potassium phosphate buffer, pH 6.8. Values are corrected volumes. Lines include error analysis.

The flavin is found to be tightly bound. Fluorescence titration by FMN followed at 525 nm with adenylate proteinoid prepared in the absence of FMN yielded a binding constant of 2×10^5. Addition of 2:1:1 butanol-1: glacial acetic acid:water releases the cofactor. The released cofactor moves indistinguishably from FMN on thin layer chromatography in this solvent. The resulting deflavo-polypeptide, however, possesses greatly altered solubility properties as a result of the butanol-glacial acetic acid-water treatment. Pronase digestion releases the flavin also.

Figure 3 shows the spectral properties of the material obtained by polymerization in the presence of hemin chloride. Two bands were normalized to equivalent absorbance to accentuate the spectral shifts.

Catalytic activity has been noted for the flavoproteinoid material as a pyruvate decarboxylase model system. Increases in rates were noted for the polymerization bound FMN upon photoillumination from an ordinary 50 W light bulb 15 cm from a Warburg flask in a recirculating Bronwill-Warburg water bath. The kinetics are shown in Figure 4. Reduction by sodium dithionite and reduced nicotinamide adenine dinucleotide were followed in a Gibson stopped-flow apparatus[14] by using a specially designed deoxygenation apparatus[15]. The light-dependent EDTA reduction under anaerobic conditons[16] was also examined. These all showed an insignificant level of enhancement of reaction rate.

This low molecular weight protein model produced enhancements of pyruvate decorboxylation reaction rate of 3–4 fold. Enhancement of peroxidase activities in thermal proteinoid have been reported as high as 50 fold[17].

Thus only small valued effects of enhancement of reaction rate are obtained in the presence of material lacking evolutionary specialization. These enhancements of reaction rate demonstrate that far more specific interactions are necessary to obtain the 8 to 13 order of magnitude enhancements of reaction rates noted in contemporary enzymes[13].

Résumé. Des modèles d'enzymes d'oxydoréduction ont été préparés par polymérisation des 20 aminoacyladénylates en présence de flavine mononucléotide, d'hème et de chlorophylle. L'addition du cofacteur flavinique au cours de la polymérisation modifie la composition en acides aminés du polymère obtenu. L'article offre une discussion sur les taux de réaction, les absorptions optiques et de dispersion rotatoire et les propriétés générales des modèles d'enzymes d'oxydoréduction.

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