In Vitro Synthesis of Procollagen on Polysomes

(collagen/protein synthesis/mRNA/initiation factors)

S. S. KERWAR*, LEONARD D. KOHN†, CHARLES M. LAPIERE‡, AND HERBERT WEISSBACH*

* Roche Institute of Molecular Biology, Nutley, New Jersey 07110; † Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014; and ‡ Department of Dermatology, Hospital de Baviere, University of Liege, Liege, Belgium 4000

Communicated by Robert W. Berliner, July 14, 1972

ABSTRACT The major collagenous products synthesized in a cell-free polysome preparation are pro- α_1 and pro- α_2 , formed in a ratio of 2:1. They are the precursor forms of α_1 and α_2 chains of normal connective-tissue collagen that are also formed in small amounts. A procollagen peptidase activity has been demonstrated in the supernatant fraction that can account for the formation of α_1 and α_2 chains from their precursors. The polysomal system is activated by a salt extract of reticulocyte ribosomes and is inhibited by aurointricarboxylic acid, suggesting that the polysomes are able to initiate protein synthesis.

Collagen molecules are composed of three polypeptide chains (2 α_1 and an α_2); each has a molecular weight of about 95,000. Using calveria systems and pulse-chase experiments, workers in two laboratories (1, 2) have demonstrated the existence of pro- α_1 . They estimate its molecular weight to be about 120,000 by gel electrophoresis in the presence of sodium dodecyl sulfate. Lenaers *et al.* (3) simultaneously recognized that a genetic recessive disease of cattle, dermatosparaxis, was associated with an inability to form normal bundles of collagen fibers and with an accumulation of unusual forms of both α_1 and α_2 chains. They demonstrated that one of the unusual forms was analogous to the pro- α_1 chain demonstrated by the calveria systems and they suggested that the second was in fact pro- α_2 . They showed that the molecular weights of pro- α_1 and pro- α_2 were 105,000 ($\pm 5\%$) and 100,000 ($\pm 5\%$), respectively, that there was an extra peptide on the amino-terminus of the pro- α_1 and pro- α_2 chains that accounted for the increased size, and that the diseased cattle accumulated these precursor forms because of a missing enzyme, "procollagen peptidase" (3, 4). They also showed that this enzyme cleaved the peptide extensions to yield normal α_1 and α_2 chains (4).

If pro- α_1 and pro- α_2 chains are the precursor forms involved in collagen biosynthesis and assembly, they should be the initial products of mRNA translation. In addition, there should be an mRNA molecule coding for each precursor form, since Vuust and Piez (6) have shown that the α_1 and α_2 chains are synthesized as single polypeptide units. Recently, Lazarides and Lukens (7) described a cell-free polysome preparation that completed and released nascent collagen chains. They used this preparation to show that the size of their active polysome fraction was consistent with that expected for a monocistronic mRNA. Since chromatography of their *in vitro* product on carboxymethyl (CM)-cellulose demonstrated an equal amount of radioactivity in the two peaks coeluting with the α_1 and α_2 chains of carrier collagen,

they initially suggested that α_1 and α_2 chains were synthesized and released in a 1:1 ratio. However, they noted that a radioactive peak eluting earlier could be pro- α_1 , that this peak contained the same amount of radioactivity as the α_1 peak, and that the α_1 and α_2 chains could be synthesized in a 2:1 ratio if the radioactivity of the presumed pro- α_1 peak and that of the α_1 peak were combined. The identity of pro- α_1 was not established in this earlier study, and no data on the formation of pro- α_2 was presented. In addition, the mechanism by which α_1 was formed remained in doubt.

In the present report, we demonstrate, in an analogous *in vitro* system, that the major collagenous products formed by the polysomal fraction are in fact pro- α_1 and pro- α_2 in a 2:1 ratio. We further show that any α_1 and α_2 chains present in the *in vitro* product can be accounted for by the presence of an active "procollagen peptidase" in the supernatant fraction and that protein synthesis is significantly activated by a salt extract of reticulocyte ribosomes that presumably contains initiation factors.

MATERIALS AND METHODS

[³H]Proline (generally labeled, 4.7 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass. Yeast tRNA was a product of Schwarz-Mann, Orangeburg, N.Y., and rabbit liver tRNA was obtained from General Biochemicals, Chagrin Falls, Ohio. A highly purified preparation of bacterial collagenase (free of nonspecific proteases) was obtained from Advance Biofactures, Lynbrook, N.Y.

Wings and legs of 100 eight-day chicken embryos were removed and homogenized in a loose-fitting Dounce homogenizer (7). Polysome fractions were prepared by layering 1.5 ml of the homogenate on a 36-ml sucrose gradient (15–40%) (7). After centrifugation at 27,000 rpm in an SW-27 rotor for 150 min, the tubes were punctured and 50% sucrose was pumped through the bottom of the tube. 1-ml fractions were collected. Fractions 1–12, 13–24, and 25–38 designated as light, medium, and heavy polysomes, respectively, were pooled and centrifuged at 265,000 $\times g$ for 3 hr. These polysomal fractions were then assayed for aminoacid incorporation into total protein and procollagen or collagen synthesis.

For preparation of an S-30 fraction, detergents were omitted from the homogenizing buffer, and the supernatant after centrifugation at 30,000 $\times g$ for 30 min was used as the source of aminoacid-activating enzymes, elongation factors, and other components required for protein synthesis.

A 0.5 M KCl wash of reticulocyte ribosomes was prepared (8). The salt wash was brought to 65% saturation with

ammonium sulfate, and the precipitate was dissolved in buffer containing 10 mM Tris·HCl (pH 7.4)–100 mM KCl–1 mM dithiothreitol–0.1 mM ethylenediaminetetraacetate (EDTA). The ammonium sulfate fraction was dialyzed against the same buffer (12 hr at 4°). This preparation is commonly used as a source of crude initiation factors.

Procollagen peptidase was a purified preparation from normal calf tendons. The enzyme preparation was about 85–95% purified as determined by analytical disc gel electrophoresis (9) and had the properties as described for crude preparations (4). The enzyme has an estimated molecular weight of 136,000 by gel electrophoresis in the presence of sodium dodecyl sulfate (10). The enzyme preparation at a concentration of 0.25 mg/ml completely converts the pro- α_1 and pro- α_2 chains of 200 μ g of dermatoparaxic collagen to normal α_1 and α_2 chains under standard assay conditions (4). The enzyme is also active with rat and chick calveria procollagen as substrate.[§]

Measurements of Aminoacid Incorporation into Protein and Procollagen–Collagen. Pellets derived from the different polysome fractions were suspended in the homogenizing buffer without detergents (7). Assays for aminoacid incorporation into protein contained in a total volume of 0.225 ml: 2–3 A_{260} units of polysome fraction, 20 mM Tris buffer (pH 7.4), 5 mM Mg^{2+} , 2 mM ATP, 0.4 mM GTP, 3 mM phosphoenolpyruvate, 0.03 μ g of pyruvate kinase, 100 mM KCl, 1 mM dithiothreitol, 0.8 mM each of a mixture of 19 amino acids (without proline), 30 μ Ci of generally labeled proline (6.3 nmol), 750 μ g each of yeast tRNA and rabbit liver tRNA, 650 μ g of protein in the S-30 fraction, and 160 μ g of protein present in the reticulocyte ribosomal salt wash. After incubation at 37° for 30 min, duplicate 0.05 ml-aliquots were removed. To one group of samples, 5 μ l of collagenase (12.5 enzyme units) were added and then both groups were incubated for an additional 30 min at 37°. Tris base (pH 10.0) (100 μ mol in 0.1 ml), was added to all samples and, after incubation for an additional 10 min at 37° to deacylate any residual [³H]prolyl-tRNA, 2 ml of cold 5% Cl_3CCOOH was added. The amount of labeled proline incorporated into the synthesized protein and in procollagen–collagen was assayed by filtration of samples through a nitrocellulose filter (0.45 μ m, Millipore Corp.). The filter was washed at least three times with cold 5% Cl_3CCOOH , dried, and assayed for radioactivity in an Omnifluor liquid scintillation fluid. The decrease in radioactivity after collagenase treatment was used as a preliminary measure of procollagen or collagen synthesis.

Analysis of the In Vitro Product by CM-Cellulose Chromatography. For analysis of the *in vitro* product, the incubation mixtures were approximately doubled. After incubation at 37° for 30 min, 0.8 ml of 1 M Tris buffer (pH 10.0) was added, and after 20 min at 37°, the mixture was centrifuged at 265,000 $\times g$ for 60 min for removal of polysomes and ribosomes. The supernatant was diluted to 2 ml with 0.02 M sodium acetate (pH 4.8) containing 1 M urea, and the sample was dialyzed against the same buffer at 4° for 12 hr. To 1

ml of the sample was added 14 ml of carrier collagen (1.5 mg/ml) that had been dialyzed against the same sodium acetate–urea buffer; the carrier collagen consisted of a 3:2 mixture of normal calf skin and dermatoparaxic calf-skin collagen (3), respectively. After denaturing at 60° for 10–30 min, samples were applied to a 2.5 $\times$ 18-cm CM-cellulose column that had been previously equilibrated at 40° with the sodium acetate–urea buffer above. The column was developed at 40° by use of a linear gradient with 400 ml of equilibrating buffer in the mixing chamber and 400 ml of equilibrating buffer containing 0.1 M NaCl in the reservoir. To elute pro- α_2 and α_2 , a second gradient consisting of 100 ml of the acetate–urea buffer containing 4 M NaCl (reservoir) and 100 ml of the acetate–urea buffer containing 0.1 M NaCl (mixing chamber) was applied. Fractions of 8.5 ml were collected and assayed for both radioactivity and absorbance at 226 nm. This procedure is a modification of that described (2, 3, 11).

Separations by Disc Gel Electrophoresis. Since pro- α_2 and α_2 cannot be separated on CM-cellulose columns, the fractions of the radioactive peak labeled pro- α_2 + α_2 were pooled and concentrated by ultrafiltration in the presence of 2–5 mg of carrier dermatoparaxic collagen. The concentrated material was subjected to disc gel electrophoresis (12) as modified by Lenaers *et al.* (3). After electrophoresis for 5 hr, the gels were treated with amido–Schwarz stain. The bands corresponding to α_2 , pro- α_2 , α_1 , and pro- α_1 were cut out manually and digested at 50° with 0.5 ml of 3% H_2O_2 for 12 hr. After addition of 0.5 ml of NCS tissue solubilizer (Amersham/Searle), the radioactivity associated with the gel bands was assayed in a toluene-based scintillation fluid (13).

Purification by Coprecipitation and Conversion of the In Vitro Products to α_1 and α_2 with Procollagen Peptidase. To 0.6 ml of the *in vitro* product (isolated and dialyzed against the 0.02 M sodium acetate–urea buffer as above) 800 μ g of calf-dermatoparaxic procollagen (1 mg/ml in 0.4 M NaCl) and 0.2 ml of 0.2 M potassium phosphate buffer (pH 7.2) were added. The procollagen was extracted and purified in a manner analogous to that described earlier (4). A 0.8-ml aliquot of a freshly made Cl_3CCOOH –phosphate solution (pH 2.5) (3, 4), was added, and after 20 min at 0°, the sample was centrifuged at 40,000 $\times g$ for 15 min. To the supernatant fluid was added 0.6 ml of cold absolute ethanol and, after 2 hr at 0°, the precipitate was collected by centrifugation at 26,000 $\times g$ for 15 min. The precipitated procollagen was solubilized in 0.3 ml of 0.1 M acetic acid. 50% of this material was chromatographed on CM-cellulose after addition of a carrier procollagen–collagen solution and dialysis in 0.02 M acetate (pH 4.8) containing 1 M urea as described above. The other 50% of the material was neutralized to pH 7.0 and digested with procollagen peptidase for 6 hr at 27° as described (4). The digested sample was mixed with carrier procollagen–collagen, dialyzed in 0.02 M acetate (pH 4.8) containing 1 M urea, and subjected to CM-cellulose chromatography.

RESULTS

By treatment with collagen proline hydroxylase and by measurement of the released tritium from [β ,4-³H]proline incorporated into the *in vitro* product, Lazarides and Lukens (6) reported that 11–14% of the protein synthesized by chick poly-

[§] These data were obtained in collaboration with Drs. P. Byers and G. Martin, National Institute of Dental Research, National Institutes of Health, and will be published in a subsequent report concerning the species specificity of the calf-tendon procollagen peptidase.

TABLE 1. Analysis of the various polysome products for radioactivity digestible with collagenase

Polysome fraction	Radioactivity retained on nitrocellulose filters	
	Without collagenase	With collagenase
1-12 (Light)	18,200	18,300
13-24 (Medium)	66,800	65,100
25-37 (Heavy)	79,000	68,600

The amount of radioactivity passing through the filters after incubation with highly purified collagenase was used as a measure of collagen synthesis, since collagen is retained on the filters under the conditions used.

somes represented collagen. They also reported that polysomes sedimenting in the heavy region were active in their preparation. The present results confirm these studies. When the three polysome fractions prepared from sucrose gradients were assayed for amino acid incorporation and their ability to synthesize collagen, only those polysomes that sedimented in the heavy region of the sucrose gradient were active in collagen synthesis (Table 1). About 15% of the radioactive proline incorporated was solubilized when previously incubated with bacterial collagenase.

The requirements for incorporation of [^3H]proline into the total synthetic product and into collagen were examined in the heavy polysome fraction (Table 2). Maximal incorporation was dependent on the S-30 fraction and on the salt wash of reticulocyte ribosomes presumably containing initiation factors. A dependency on tRNA could not be demonstrated. When aurintricarboxylic acid was added to the complete preparation, incorporation of proline was diminished to about the amount that was obtained when the ribosomal salt wash was omitted from the incubation mixture. Recent studies (14-16) have shown that aurintricarboxylic acid inhibits eukaryotic protein synthesis by inhibiting chain initiation, whereas it has no effect on elongation of the nascent peptide chains. The stimulation obtained by addition of the salt wash of ribosomes and the sensitivity of the incorporation of [^3H]proline to aurintricarboxylic acid provide presumptive evidence that the polysomes are able to initiate protein synthesis.

Analysis of the *In Vitro* Product. Fig. 1A shows the results of analysis of the total *in vitro* product by CM-cellulose chromatography. Fig. 1B shows the result of a similar analysis of the *in vitro* product after a preliminary purification by coprecipitation with dermatosparaxic collagen. Fig. 1C shows the results after a preliminary precipitation with dermatosparaxic collagen followed by digestion with procollagen peptidase. It is clear from these data that a major peak of radioactivity cochromatographs with carrier pro- α_1 (Fig. 1A and B)

¶ Previous data have recorded two peaks of pro- α_1 in dermatosparaxic collagen samples, the second peak being a variable finding from preparation to preparation (3). The CM-cellulose data of the total *in vitro* product (Fig. 1A) has a radioactive peak coincident with this second pro- α_1 species; the CM-cellulose data of the *in vitro* product preliminarily purified by coprecipitation with dermatosparaxic collagen do not. At present, there is no explanation for this variable finding.

TABLE 2. Requirements for incorporation of [^3H]proline into the synthetic products of the heavy-polysome fraction

Preparation	pmol of proline incorporated into	
	total protein	collagen
Complete	8.4	1.2
-S-30	1.2	0.3
-initiation factors	1.4	0.3
-tRNA	8.2	1.1
Complete + 0.2 mM aurintricarboxylic acid	1.5	0.4

The total volume of each incubation mixture was scaled down to 75 μl and contained 0.42 A_{280} units of the heavy-polysome fraction. The amount of radioactive proline incorporated in the absence of polysomes (1.26 pmol) was used as a blank and was deducted from each value. Incubations were for 30 min at 37°.

and that the radioactivity associated with this peak is converted by procollagen peptidase to material that cochromatographs (Fig. 1C) with the α_1 standard.

Since CM-cellulose chromatography does not resolve pro- α_2 and α_2 , the radioactive material eluting in the pro- α_2 + α_2 region (Fig. 1A) was pooled, concentrated, and subjected to polyacrylamide gel electrophoresis in the presence of dermatosparaxic procollagen. As shown in ref. 3 and in Fig. 2, this procedure resolves pro- α_2 from α_2 chains. It is clear from Fig. 2A that the radioactive product eluted in the pro- α_2 + α_2 region of the CM-cellulose eluate is predominantly pro- α_2 and that after exposure to procollagen peptidase, the radio-

FIG. 1. CM-cellulose chromatography of 1.0 ml of the total *in vitro* product (A), of 0.3 ml of the product after preliminary coprecipitation with dermatosparaxic procollagen (B), and of 0.3 ml of the product after both preliminary coprecipitation and procollagen peptidase digestion (C). Total cpm applied was 240,000, 12,400, and 12,400 respectively (see Methods). Peaks are designated on the basis of disc gel electrophoresis of both the labeled product and an unlabeled standard dermatosparaxic procollagen (5) (Fig. 2). The black bars denote fractions that were pooled to obtain the data of Fig. 2 and Table 4.

FIG. 2. Identification of labeled products by disc gel electrophoresis. The radioactive peaks of Fig. 1 were concentrated in the presence of carrier dermatosparaxic procollagen (see *Methods*), and were subjected to disc gel electrophoresis before and after digestion with procollagen peptidase. Gels were stained with amido-Schwartz and cut into slices such that each species (α_2 , pro- α_2 , α_1 , and pro- α_1) was separated by visual inspection of the dye patterns. Each slice was treated with H_2O_2 and its radioactivity was measured (13). About 750 cpm were applied to each gel; average recoveries in all cases were about 55% of the material applied. Frame A demonstrates that the pro- α_2 - α_2 peak is predominantly pro- α_2 before digestion, but predominantly α_2 afterward. Frame B shows that radioactive peak labeled pro- α_1 (Fig. 1A) is predominantly pro- α_1 before digestion but is converted to α_1 after treatment with procollagen peptidase.

active product is converted to material that coelectrophoreses with standard α_2 . It is also clear from Fig. 2B that the radioactivity eluting in the pro- α_1 (Fig. 1A) area is predominantly pro- α_1 and is converted to material that coelectrophoreses with α_1 after digestion with procollagen peptidase.

It was now possible to quantitate the *in vitro* products more thoroughly. About 15% of the synthetic product behaves as collagenous material (collagen or procollagen), according to its collagenase sensitivity or coprecipitability with dermatosparaxic or normal collagen as summarized in Table 3. Table 4 summarizes the data obtained in Figs. 1 and 2 on the ratio of pro- α_1 to pro- α_2 formed. It can be seen that the pro- α_1 and pro- α_2 chains are synthesized in a 2:1 ratio.

Although these data show that pro- α_1 and pro- α_2 chains are the major collagenous *in vitro* products, some collagenase-sensitive radioactivity does elute with α_1 and α_2 (Fig. 1). It seemed reasonable that this could be due to the presence of procollagen peptidase in the tissue extract. This was con-

firmed by analysis of the S-30 fraction. A procollagen peptidase activity, sensitive to EDTA, was readily detected (Fig. 3). Since the pH of the *in vitro* incubations (7.4) is optimal for the action of this enzyme, one can readily explain the presence of the α_1 and α_2 chains as reaction products.

TABLE 4. Product radioactivity recovered as precursor (pro- α_1 , pro- α_2) or normal (α_1 , α_2) collagen chains (cpm)

	The total synthetic mixture (from Fig. 1A)	After coprecipitation with dermatosparaxic collagen (from Fig. 1B)	After coprecipitation with collagen and after digestion with procollagen peptidase (from Fig. 1C and Fig. 2)
pro- α_1	18,700	5,500	740
α_1	3,900	1,200	6,400
α_2 + pro- α_2	10,200	3,200	3,600
α_2	1,950	600	3,200
pro- α_2	8,250	2,600	400

TABLE 3. Collagen content of the *in vitro* product synthesized by the heavy-polysome fraction

Treatment	Before treatment (cpm of the total <i>in vitro</i> product)	After treatment (cpm as collagen or pro-collagen)	% radioactivity incorporated into procollagen or collagen
Collagenase digestion*	79,000	10,300	13.1
Coprecipitation†			
With normal calf collagen	24,500	3,970	16.2
With dermatosparaxic calf collagen	24,500	3,530	14.4

* See also Table 1.

† See *Methods*.

Total cpm applied to column (Fig. 1A) was 240,000. In Fig. 1B and C, 0.6 ml (144,000 cpm) of a product mixture analogous to that in Fig. 1A was coprecipitated with dermatosparaxic collagen (see *Methods*) and divided into two equal aliquots containing 12,350 cpm each. One was applied directly to a CM-cellulose column and one was applied after digestion with procollagen peptidase (see *Methods*). The fractions containing pro- α_1 , α_1 , and pro- α_2 - α_2 were individually pooled (black bars, Fig. 1) and quantitated. In all cases, the amount of radioactivity in pro- α_1 + α_1 compared to that in pro- α_2 + α_2 approached a 2:1 ratio. The allocation of the pro- α_2 - α_2 radioactivity to each species, pro- α_2 or α_2 , is based on the assumption that the α_2 component is 50% of the α_1 component from the same experiment and on the data of Fig. 2.

FIG. 3. Procollagen peptidase activity of the S-30 fraction used in the protein-synthesis mixture. Gels 1, 2, and 3 are, respectively, control dermatosparaxial procollagen, dermatosparaxial collagen plus S-30 fraction (500 μ l), dermatosparaxial collagen plus S-30 fraction (500 μ l) plus EDTA, 25 mM. All were incubated under optimal assay conditions for procollagen peptidase activity (4). Incubations lasted 24 hr. The second gel electrophorogram shows the loss of the pro- α_1 and pro- α_2 bands and their conversion to material that coelectrophoreses with α_1 and α_2 . The third gel demonstrates the inhibition of this proteolytic activity by EDTA.

DISCUSSION

In the present experiments data are presented that indicate that pro- α_1 and pro- α_2 chains are the major collagenous, and very likely initial, products synthesized on the polysome and that they are synthesized in a 2:1 ratio. The presence of small amounts of α_1 and α_2 chains in the incubation mixtures is very likely due to the presence of procollagen peptidase in the S-30 fraction. Identification of pro- α_1 and pro- α_2 is based on the analogous behavior of the radioactive product and the pro- α_1 and pro- α_2 chains of dermatosparaxial-calf collagen (3) before and after exposure to procollagen peptidase (4).

Recently Church, Pfeiffer, and Tanzer (17) have postulated that the precursor form of collagen is a high molecular weight complex that is digested to yield the triple helical collagen structure of normal connective tissues. The present data, and those of Lazarides and Lukens (7), Vuust and Piez (6), and Dehm *et al.* (5), suggest that the initially synthesized precursors are pro- α_1 and pro- α_2 , and that the high molecular weight complex noted in fibroblasts (5, 17-19) is an aggregated form of pro- α_1 and pro- α_2 . This aggregate appears to be stabilized by disulfide bonds and might well be the form of

collagen transported within and secreted from the cell (5, 18, 19). In this regard, it is interesting to note that procollagen peptidase activity is readily assayed in the media of fibroblast cultures rather than in the cells (unpublished data), that procollagen peptidase is more active on polymerized and aggregated forms of the pro- α_1 and pro- α_2 chains than on monomer forms (unpublished data), and that the presence of pro- α_1 influences the catalytic conversion of pro- α_2 to α_2 (4). Procollagen peptidase might, thus, function extracellularly to cleave these "transport aggregates" before polymerization into collagen fibers.

1. Bellamy, G. & Bornstein, P. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1138-1142.
2. Müller, P. K., McGoodwin, E. & Martin, G. R. (1971) *Biochem. Biophys. Res. Commun.* **44**, 110-117.
3. Lenaers, A., Ansay, M., Nusgens, B. V. & Lapiere, C. M. (1971) *Eur. J. Biochem.* **23**, 533-543.
4. Lapiere, C. M., Lenaers, A. & Kohn, L. D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 3054-3058.
5. Dehm, P., Jimenez, S. A., Olsen, B. R. & Prockop, D. J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 60-64.
6. Vuust, J. & Piez, K. A. (1970) *J. Biol. Chem.* **245**, 6201-6207.
7. Lazarides, E. & Lukens, L. N. (1971) *Nature New Biol.* **232**, 37-40.
8. Miller, R. L. & Schweet, R. (1968) *Arch. Biochem. Biophys.* **125**, 632-641.
9. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404-427.
10. Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328-6334.
11. Piez, K. A., Eigner, E. A. & Lewis, M. S. (1963) *Biochemistry* **2**, 58-66.
12. Sakai, T. & Gross, J. (1967) *Biochemistry* **6**, 518-528.
13. Tishler, P. V. & Epstein, C. J. (1968) *Anal. Biochem.* **22**, 89-98.
14. Marcus, A., Bewley, J. D. & Weeks, D. P. (1970) *Science* **167**, 1735-1736.
15. Lebleu, B., Marbaix, G., Werenne, J., Burny, A. & Huez, G. (1970) *Biochem. Biophys. Res. Commun.* **40**, 731-737.
16. Stewart, M. L., Grollman, A. P. & Huang, M. T. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 97-101.
17. Church, R. L., Pfeiffer, S. E. & Tanzer, M. L. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2638-2642.
18. Layman, D. L., McGoodwin, E. B. & Martin, G. R. (1970) *Fed. Proc.* **29**, 668 Abstr.
19. Layman, D. L., McGoodwin, E. B. & Martin, G. R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 454-458.