TABLE II. Analyses of Hydrolyzed Pooled Plasma.

No.	Date specimen obtained and filtrate made	Date of analyses (1964)	Method of storage, °C	Glutamine	Asparagine
				Plasma conc, mg/100 ml	
1	July 17, 1964	7/20	4	11.9 12.0	2.83 2.89
2		31	−70	11.2 11.3	2.97 3.48
3		9/29	4	11.3	3.41
4		30	−70	11.3	3.36
5		20	4	12.1	3.48
6		10/20	−70	11.4	3.06
7		27	−70	11.6	3.51

Plasma filtrate stored at 4°C and −70°C for about 3 to 4 weeks revealed no increase in glutamic acid, confirming the suggestion that deproteinized physiologic solutions are stable (Table I). Studies have not been performed to evaluate whether these stored filtrates contain pyrrolidone carboxylic acid and ammonia.

Summary. The data presented indicate that glutamine remained stable when prepared in citrate or aqueous solution, stored at 4°C for at least 8 weeks or at room temperature for 3 to 4 weeks. Analyses of deproteinized plasma indicate that the glutamic acid concentrations were constant and stable for approximately 4 weeks. Plasma glutamine determinations were also quantitatively reproducible with time. These results would also suggest that with deproteinization of blood, the values obtained for glutamic acid are not due to glutamine breakdown but are true glutamic acid values.

ADDENDUM: Since submitting this manuscript, it has been demonstrated that there is no significant increase in ammonia concentration in samples analyzed for one week from a plasma filtrate.

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Inhibition of Proline-C^{14} Incorporation into Rat Liver Ribosomes by Thiazolidine-4-Carboxylic Acid in a Cell-Free System.* (30295)

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The hydroxyproline in collagen-like proteins synthesized *in vitro* has been shown to originate from free proline(1). The question then arises as to whether proline is being converted to hydroxyproline by way of a transfer RNA (t-RNA) bound proline inter-

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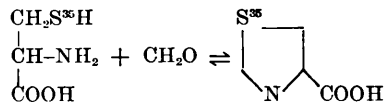
mediate(2), or a microsomal bound peptide rich in proline(3). The use of a specific inhibitor to proline incorporation into protein would be of some significance in elucidating the mechanism of collagen biosynthesis in a cell-free system. The following experiments establish the mechanism of inhibition of proline incorporation into ribosomes by thiazolidine-4-carboxylic acid.

Thiazolidine-4-carboxylic acid (TZCA), an analog of proline, has been shown to be metabolized *in vivo*(4). It is oxidized by rat liver mitochondria(5), and it is also converted to cysteine and cystine, *via* N-formylcysteine in the intact animal(6). Hence, *in vivo* studies would not lead to any significant inhibition of proline incorporation into proteins, because TZCA can easily be metabolized and removed from the amino acid pool. The experiments reported here show that TZCA manifests an inhibitory effect on the incorporation of proline-C^{14} into ribosomes when protein synthesis is studied in a cell-free system.

Materials and methods. Synthesis of TZCA-S^{35}. TZCA was synthesized according to the method described by Ratner and Clarke (7). The method is outlined in equation below. A total of 69 mg of DL-cysteine-S^{35} hydrochloride (3 mc) were mixed with 431 mg of



unlabelled L-cysteine hydrochloride, and dissolved into 1.5 ml of distilled water; 0.37 ml of commercial 38% formaldehyde was added, and the mixture allowed to stand overnight at room temperature. Crystals were separated on the addition of 0.83 ml of pyridin. The mixture was diluted with 0.8 ml of 95% ethanol, and centrifuged. The precipitate was recrystallized from hot water (85°C). A yield of about 50% was obtained. The long, colorless needles were dissolved in 15 ml of distilled water. The specific activity of this preparation was 0.5 $\mu c/\mu mole$ in DL-TZCA-S^{35}. Further purification, when necessary, was performed by paper chromatography using phenol-O-cresol (1:1) as the developing system. TZCA gives a yellow spot when the chromatogram is sprayed with ninhydrin. Proline and hydroxyproline are known to give this type of color when reacted with ninhydrin. The R_f values are reported in Table I. A developed chromatogram of TZCA-S^{35}, scanned with a strip counter supplemented by autoradiography, showed one peak only coinciding with that of TZCA. Analysis on a Beckman amino acid analyzer model 120B, also exhibited a single peak corresponding to that of TZCA. Hence, the purity of TZCA-S^{35} was 100% following 2 recrystallizations from hot water or purification on paper.

TABLE I. R_f Values of the Various Amino Acids Developed by Paper Chromatography, Using Phenol-O-Cresol (1:1) as the Descending System.

Amino acid	R_f value
Cysteine	0
Hydroxyproline	.480
TZCA	.624
Proline	.780

Preparation of ribosomes and pH 5 enzymes. Livers were excised from rats weighing 150 to 300 g which were fasted for 16 to 20 hours. The livers were weighed, placed in 2 vol/g of cold solution A (0.005 M $MgCl_2$, 0.002 M KCl, 0.15 M sucrose, 0.005 M mercaptoethanol, in 0.1 M Tris-HCl, pH 7.5), and homogenized for 15 seconds at medium speed in a Virtis homogenizer. The homogenate was centrifuged at 15,000 $\times$ *g* for 25 minutes in a Spinco model L ultracentrifuge. The supernatant was aspirated and centrifuged at 105,000 $\times$ *g* for 90 minutes. The pH 5 enzymes were prepared from the S-105 supernatant as previously described(8). The microsomal pellet, representing about 20 g of liver, was suspended into 5 ml of solution B (0.005 M $(CH_3COO)_2Mg \cdot 4H_2O$, 0.025 M KCl, 0.005 M mercaptoethanol, in 0.05 M Tris-HCl, pH 7.5), to which 1.0 ml of 5% sodium deoxycholate (DOC) was added. The volume of the suspension was then brought up to 10 ml with solution B, yielding a final concentration of 0.5% DOC. The mixture was first centrifuged for 10 minutes at 10,000 $\times$ *g* to remove aggregates, then recentrifuged at 105,000 $\times$ *g* for 90 minutes in the S-40 rotor. After centrifugation the DOC treated

particles (ribosomes) were suspended in solution B and recentrifuged for 90 minutes. The washed ribosomal pellet was resuspended in solution B and kept at 0°C until used, or stored overnight at —75°C. Particle protein was determined by the method of Lowry *et al*(9).

Incorporation assay. The complete reaction mixture in 1.0 ml consisted of 0.5-0.8 mg of pH 5 enzymes, 1.0-1.5 mg of ribosomal protein, 5 μmoles Mg^{++}, 50 μmoles KCl, 2 μmoles ATP,§ 5 μmoles of creatine phosphate, 0.025 mg of creatine phosphokinase, 0.5 μmole GTP, 0.1 μmole each of 18 unlabelled amino acids, in 0.1 M Tris-HCl, pH 7.5, containing 0.005 M mercaptoethanol, and 1 μc of UL-proline-C^{14} (specific activity, 180 μc/μmole), or 500,000 cpm of TZCA-S^{35}. The mixture was incubated at 37°C in a Dubnoff shaking water bath under air. After incubation, the reaction was stopped with 4 ml of 5% trichloracetic acid, and the resulting precipitate was washed and analyzed by the Siekevitz procedure(10). The washed precipitate was dissolved into 0.5 ml of hyamine and analyzed for radioactivity in a Nuclear-Chicago scintillation spectrometer, in toluene containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)benzene. Specific activity was calculated and is expressed as counts/min/mg of ribosomal protein. Zero time incorporation was subsracted from all incorporation data.

Preparation of transfer RNA-TZCA-S^{35}. Transfer RNA-TZCA-S^{35} was prepared by the method of Zamecnik *et al*(11). The amino acid was cleaved from t-RNA as previously described(11), and identified by paper chromatography as described under synthesis of TZCA-S^{35}.

Studies with poly C§. Poly C was used as the synthetic messenger RNA, which is known to code for the incorporation of proline into ribosomes(12), to find out if it can also affect the incorporation of TZCA-S^{35} into ribosomal polypeptides. To deplete endogenous messenger RNA the reaction mixture was incubated for 20 minutes prior to addition of poly C and the labelled imino acid. Different concentrations of poly C were then supplemented together with either proline-C^{14} or TZCA-S^{35}, and reincubated for an additional period of 20 minutes. Incorporation was then analyzed for as described above.

§ The following abbreviations are used: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; C, the base cytosine; poly C, polycytidylic acid; RNA, ribonucleic acid; t-RNA, transfer RNA.

TABLE II. Effect of Cofactors on Incorporation of TZCA-S^{35} by Rat Liver Ribosomes–pH 5 Precipitate Preparation.

Addition or omission	Specific activity	% Inhibition
Control	539	—
— ATP, creatine phosphate, creatine phosphokinase	436	19.1
— GTP	254	52.9
— CTP (0.5 μmole/ml)	574	.0
— Amino acids	460	14.6
— pH 5 enzymes	247	54.2
— ribosomes	137	74.9
+ puromycin (50 μg/ml)	255	52.6
+ ribonuclease (5 μg/ml)	63	88.2

Materials. ATP, GTP, and CTP were purchased from Pabst Laboratories; labelled amino acids from New England Nuclear; DL-cysteine-S^{35} hydrochloride (specific activity, 7 mc/mmole), creatine phosphate and creatine phosphokinase from CalBiochem; puromycin from Nutritional Biochemicals Co., and poly C from Mann Assay.

Results and discussion. The time study for incorporation of TZCA-S^{35} into ribosomes is shown in Fig. 1. An incubation period of 60 minutes was necessary to obtain maximum incorporation. The cofactors required for this incorporation are shown in Table II. The energy generating system, GTP, amino acids, and the ribosomal particles were required for maximum incorporation. Addition of puromycin (50 μg/ml) inhibited the incorporation of TZCA-S^{35} by 52%. Puromycin, which is known to inhibit the incorporation of t-RNA-bound amino acid into ribosomal-polypeptide (13), presents a supporting evidence that TZCA-S^{35} is incorporated into polypeptides. The addition of ribonuclease inhibited the binding of TZCA-S^{35} to ribosomes by 88%. This suggests that intact polysomes are required for activity, since ribonuclease digests endogenous messenger RNA(14). The isola-

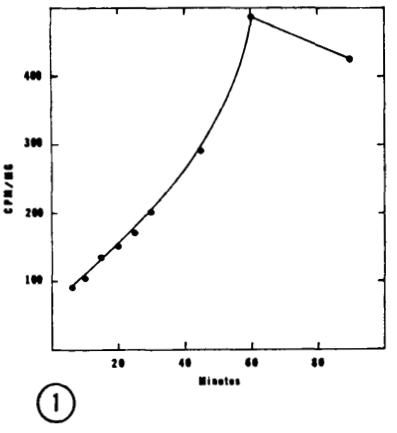
TABLE III. Synthesis of t-RNA-TZCA-S^{35}, and Identification of Labelled Amino Acid Bound to t-RNA.

cpm/mg t-RNA	R_f of recovered labelled amino acid
257	.62

tion of t-RNA-TZCA-S^{35} and identification of TZCA-S^{35} as the imino acid bound to t-RNA (Table III), ascertains that TZCA-S^{35} is incorporated into ribosomes following the formation of t-RNA-TZCA-S^{35}. In a second series of experiments, poly C, which codes for proline incorporation into ribosomes, was used to study its effect on TZCA-S^{35}. The results are shown in Fig. 2. It is seen that in addition to proline, poly C also enhances the incorporation of TZCA-S^{35} into trichloroacetic acid insoluble material. This is significant because it demonstrates that TZCA can be incorporated into ribosomes through a pathway similar to that of proline. Hence, TZCA has a code triplet which contains 3 cytidines (CCC). This imino acid analog can, therefore, specifically inhibit the incorporation of proline into proteins as shown in Fig. 3 and in Table IV. As the concentration of TZCA is increased proline incorporation is decreased (Fig. 3), which implies a competitive type of inhibition. This inhibition seems to be specific because TZCA does not affect the incorporation of lysine-C^{14} or tryptophan-H^3 into ribosomes (Table IV).

The above data bring up two significant findings: (1) TZCA-S^{35} is incorporated into proteins, and (2) TZCA-S^{35} is incorporated *via* a pathway similar to that of proline. TZCA, therefore, may be used as a competitive inhibitor to proline incorporation into ribosomes in a cell-free system. The use of such an inhibitor can advance the study on the biosynthesis of collagen in a cell-free system since it should also inhibit the formation of hydroxyproline. At this time, further experiments are being performed to explore this possibility.

Summary. Thiazolidine-4-carboxylic acid (TZCA) was used to inhibit the incorporation of proline into rat liver ribosomes in a cell-free system. It was found that TZCA was incorporated into ribosomes *via* a pathway



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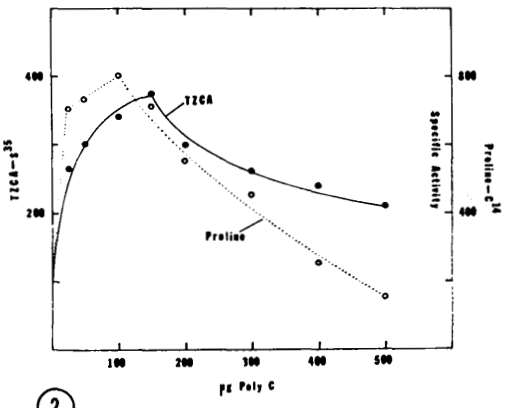

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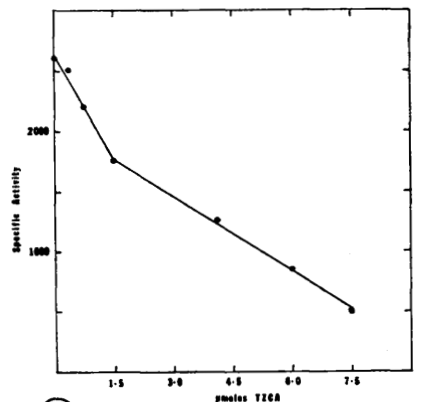

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FIG. 1. Time course study of TZCA-S^{35} incorporation into ribosomes.

FIG. 2. Effect of various concentrations of poly C on incorporation of TZCA-S^{35} or proline-C^{14} into ribosomes.

FIG. 3. Inhibition of proline-C^{14} incorporation into ribosomes by various concentrations of TZCA.

TABLE IV. Specificity in Inhibition of Proline-C^{14} Incorporation into Ribosomes by TZCA.

Amino acid	Specific activity No TZCA	Specific activity + 3.75 μmoles TZCA	% inhibition
L-lysine-C^{14} (specific activity, 220 μc/μmole)	1100	1248	.00
DL-tryptophan-2,3-H^3 (specific activity, 200 μc/μmole)	800	790	1.25
L-proline-C^{14} (specific activity, 180 μc/μmole)	2600	1250	52.0

similar to that of proline. The coding polynucleotide for this incorporation was a homopolymer of cytidine. t-RNA-TZCA-S^{35} was also isolated, and this presented further evidence that TZCA-S^{35} was incorporated into ribosomes following the formation of t-RNA-TZCA-S^{35}. Hence, these experiments show that TZCA may be used as a competitive inhibitor to the incorporation of proline into ribosomes in a cell-free system.

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Characterization of the Basic Protein Associated With DNA in Mammalian Spermatozoa.* (30296)

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The basic proteins isolated from the nucleoprotein of salmon and sturgeon spermatozoa are protamines. A more complex and less basic protein, histone, is associated with DNA in somatic cell nuclei, such as calf thymus and chicken erythrocytes(1). The histones contain arginine and lysine in a ratio ranging from 0.5-1.5:1.0(2) and no cystine, except as a contaminant(3). Besides serving a structural purpose in the chromosome(4), histones have been hypothesized to serve as regulators of gene action(5). Evidence has been presented which indicates that DNA in these genes associated with histone is prevented from expressing itself(6).

In contrast to what is known about the basic proteins of other cells, there is a disturbing lack of knowledge about the basic protein associated with DNA in mammalian spermatozoa. This is due in some measure to the existence of a keratin-like protein in this cell which makes extraction of basic protein difficult. Green(7) presented evidence for the presence of a keratin membrane coating the head of the ram spermatozoon. Other work presented evidence for a keratin-like

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