

UCSF

UC San Francisco Electronic Theses and Dissertations

Title

Alteration of protein synthesis in cartilage by a synthetic double-stranded polyribonucleotide

Permalink

<https://escholarship.org/uc/item/03z7058x>

Author

Wadell, Marcia Clair Loechler

Publication Date

1974

Peer reviewed|Thesis/dissertation

ALTERATION OF PROTEIN SYNTHESIS IN CARTILAGE BY A
SYNTHETIC DOUBLE-STRANDED POLYRIBONUCLEOTIDE

by

Marcia Clair Loechler, Wadell
B.A., University of California, Berkeley 1968

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

(San Francisco)

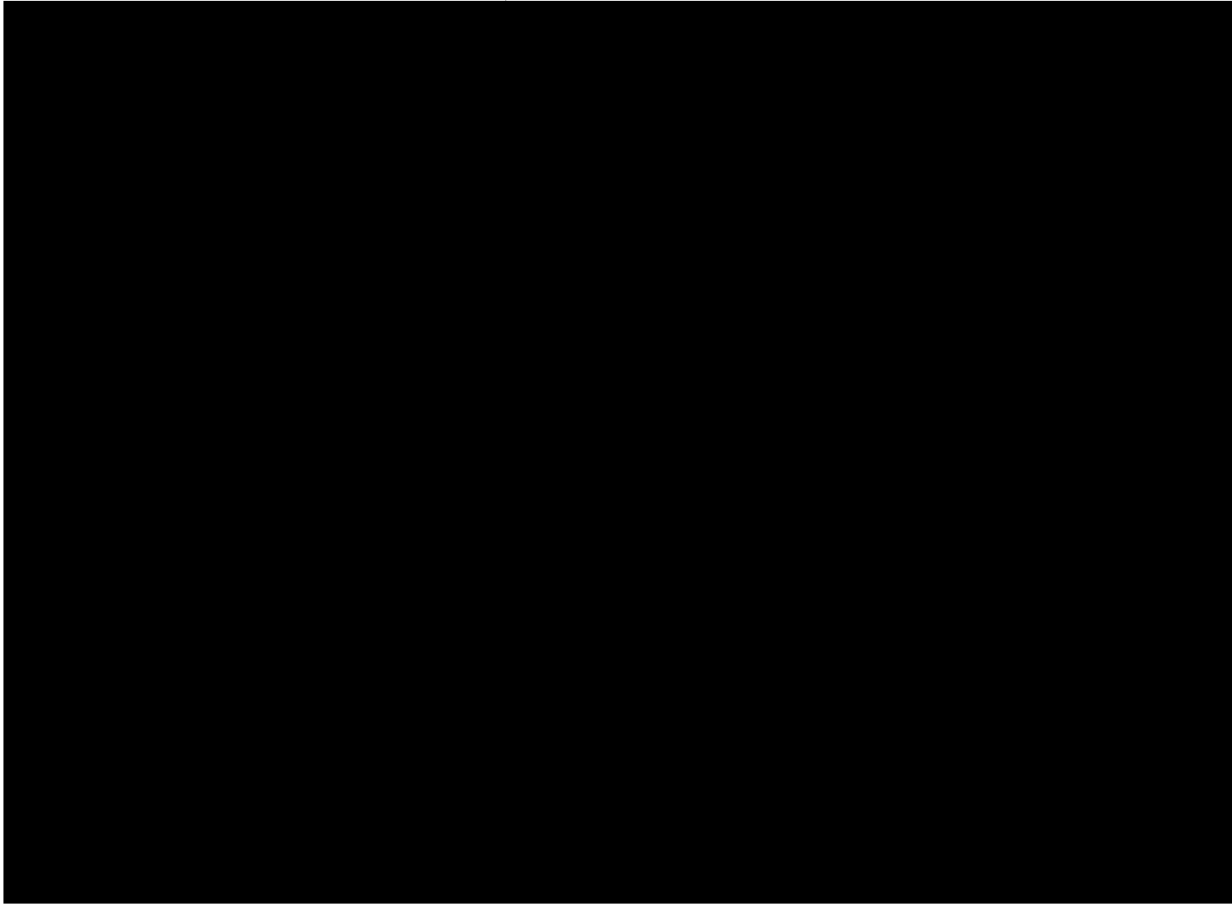


TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
TABLES	ii
FIGURES	iii
INTRODUCTION	1
MATERIALS AND METHODS	7
RESULTS	11
DISCUSSION	30
SUMMARY AND CONCLUSION	36
BIBLIOGRAPHY	38

ACKNOWLEDGEMENTS

To Dr. Rajendra S. Bhatnagar for providing his valuable time and energy that provided the inspiration and guidance through the course of my graduate study.

To Dr. Dov Michaeli, who served on my thesis committee and gave me considerate help and encouragement.

To Dr. Richard A. Fineberg, who served on my thesis committee, and as my graduate advisor.

To Jan Grohe, for her hours of expert secretarial help.

Portions of this text were presented at the International Association of Dental Research meetings in Chicago, March 1971, and received the Hatton Award, second place, predoctoral division. This paper was subsequently published in the Journal of Dental Research, 50, 1072 (1971).

TABLES

Table	Page
I. The effect of Poly IC on the synthesis of collagenase-hydrolyzable material.....	13
II. The effect of Poly IC on collagen biosynthesis.....	15
III. The statistical analysis of Figure 1.....	18
IV. The statistical analysis of Figure 2.....	21
V. The statistical analysis of Figure 3.....	23
VI. The statistical analysis of Figure 4.....	26
VII. The statistical analysis of Figure 5.....	29

FIGURES

Figure	Page
1. Time course of the stimulatory effect of Poly IC on proline incorporation in cartilage.....	17
2. The effect of Poly IC on phenylalanine incorporation into cartilage.....	20
3. The effect of Poly IC on proline incorporation in the presence of metabolic inhibitors.....	22
4. The effect of Poly IC on uridine incorporation.....	25
5. The time course of disappearance of the stimulatory effect of Poly IC, after Poly IC is removed from the medium.....	28

INTRODUCTION

Regulation of protein synthesis in eukaryotic organisms is rendered complicated by the potential among individual cells for the synthesis of a vast variety of proteins and by the suppression of a large part of this potential as a prerequisite to cellular differentiation. Interestingly, cellular differentiation also provides an effective means of investigating regulatory controls utilizing the property of some differentiated cell types to produce large amounts of one protein, often a well characterized one.

Ten-day old embryonic chick cartilage is a tissue actively engaged in the production of the scleroprotein, collagen. Collagen accounts for more than fifty percent of the total protein output in this tissue. The three polypeptide chains that make up the collagen molecule each contain about 1000 amino acids. Denaturation and chromatography these three chains yield two kinds of polypeptide chains designated as α_1 and α_2 in the ratio of 2:1. In the native form each individual chain is coiled in a tight left handed helix. The three chains, designated as $(\alpha_1)_2 \alpha_2$ are combined in a loose right handed helix producing a rod-like molecule, called tropocollagen, about 3000 Å long and 15 Å in diameter with a molecular

weight of approximately 300,000 (1-3). The amino acid sequence of the individual chains consists largely of triplets of the form $(\text{GLY-X-Y})_n$, where X and Y can be any amino acid, but it is often proline and hydroxyproline. The presence of these amino acids in large amounts, approximately 25% of the collagen molecule, tend to prevent rotation around their peptide bonds and thereby impart rigidity to the chains.

The synthesis of collagen involves several additional enzymic steps following the synthesis on the polyribosomes and subsequent release of the polypeptide chains. In the first series of enzymic reactions, several specific proline and lysine residues are hydroxylated to hydroxyproline and hydroxylysine (4-7). Most of the hydroxylation occurs after the polypeptide chains have been completed and released from the polysomal complexes (8-10), and perhaps coiled in the triple helical form (11), although it may be initiated while the nascent chains are still associated with the polyribosomes (12,13). The unhydroxylated precursor of collagen has been termed procollagen (15). Inhibition of hydroxylation does not stop amino acid incorporation, but, it blocks the secretion of collagen which accumulates intercellularly (16). Only lysine and proline found in the Y position of $(\text{GLY-X-Y})_n$ can be hydroxylated (17,18), however, not every proline and lysine in this position are hydroxylated (19,20). The hydroxylating enzymes, procollagen-proline hydroxylase

and protocollagen-lysine hydroxylase require O_2 (21), α -ketoglutarate (22), ascorbic acid (23) and Fe^{++} .

The hydroxylation of proline and lysine is followed by the glycosylation of certain hydroxylysine residues in which a galactose and a glucose residue are successively bound to give rise to glucosyl-galactosyl-O-hydroxylysine (25,26). Again, hydroxylysine can only be found in the Y position of the repeating tripeptide unit and not all the hydroxylysine residues in this position glycosylated. It appears that arginine must be in the Y position of the following tripeptide unit if hydroxylysine is to be glycosylated (27). Indirect evidence has led to the suggestion that the glycosylation step may be related to the secretion of collagen (28,29).

In recent years, a large amount of information on the mechanism of collagen biosynthesis has accumulated, but at present the knowledge of mechanisms involved in the regulation of collagen is limited. It has been suggested (30) that there may be control mechanisms which operate at a step subsequent to the transcription of collagen m-RNA in rapidly growing embryonic tissues, similar to post-transcriptional control mechanisms found in rat hepatoma cells in culture (31).

Information concerning the mechanisms of regulation of collagen synthesis is of great interest because of the involvement of aberrant synthesis of this protein in numerous pathologic conditions. Overproduction and

deposition of fibrous collagen, has been implicated in such diverse disorders as arteriosclerosis (32), cirrhosis, and in pulmonary disorders produced by breathing air polluted with particulate or gaseous contaminants (33,4).

In order to obtain insight into the mechanisms involved in the regulation of protein synthesis it would be helpful to obtain reproducible experimental conditions which would alter the rate of protein synthesis.

These investigations were prompted by an earlier observation suggesting alteration of cellular metabolism in vivo and in vitro by Poly IC (34-39). Poly IC was found to increase cellular resistance to bacterial infection (40), to inhibit the growth of viral and nonviral tumors (41,42), to accelerate homograft rejection (43) and to enhance the immunological response (44,45), both the humoral (46,47) and the cell mediated response (47,48).

Considerable interest has arisen in possible therapeutic and prophylactic applications of Poly IC (49) since it was found to be a potent inducer of viral resistance, presumably through stimulation of interferon synthesis (34). The mechanism of action of the effect of interferon inhibition of viral resistance is not completely understood. Published results seem to indicate that interferons do not directly inactivate virus particles (50,51) nor do they inhibit virus adsorption, penetration, or uncoating (52). Synthesis of both viral RNA and protein is

inhibited in cells pretreated with interferon, but this inhibition is not seen if host RNA and protein synthesis are eliminated during the pretreatment (53). Thus, it appears that interferon causes a stimulation of synthesis of an antiviral protein. This antiviral protein may act by inhibiting the translation of viral messenger RNA (53-55). The antiviral substance has been partly characterized physically and chemically. It has been shown that both viral and nonviral agents, as Poly IC, could elicit the production of interferon. It is known that there are two fundamentally different mechanisms for its production, via release of interferon from pre-formed pools (usually by nonviral agents in intact animals) (56-58) and by induction, a process involving the requirement for de novo RNA and protein synthesis (59,60). Yet, the molecular basis for both the induction of interferon and the action of interferon remained a mystery.

Since Field (38) discovered that Poly IC induces interferon in rabbit cells in culture, there have been confirmatory reports from many laboratories. The cell systems differ from each other with respect to the amount of Poly IC required to induce detectable levels of interferon. This is probably due to at least two factors: first, the capacity of the cells to take up the inducer may differ; and second, the capacity of the cells to recognize and respond to the inducer may also differ.

There is enough evidence to show that polyribo-

nucleotides can be taken up by mammalian cells (61,62) and that part of the RNA taken up is transported into the cells (63,64). The uptake of polyribonucleotides proceeds linearly, either right from the beginning or after a short lapse, and continues in this fashion for several hours (65-68). There have been very few attempts to determine what proportion of the RNA taken up by the cells stays bound to the cell surface or penetrates into the cells (69,70).

Observations in the laboratory of Dr. H. B. Levy at National Institutes of Health (71) with in vivo and tissue culture systems, and subsequent experiments in our laboratory with tissue explants in vitro demonstrated a marked alteration of protein synthesis in cartilage by the synthetic double stranded ribonucleic acid polyinosinic:polycytidylic acid (Poly IC). Since a major part of the protein synthetic activity in cartilage is directed towards the synthesis of collagen, it was of interest in order to obtain information concerning possible involvement of regulatory phenomena.

MATERIALS AND METHODS

MATERIALS

The Poly IC (1.0 mg/ml in phosphate buffered saline containing Mg^{++}) was kindly furnished by Dr. Hilton B. Levy. L-[3,4- ^3H]-proline (specific activity 5 Ci/mM), [5- ^3H]-uridine (specific activity 26.2 Ci/mM), and L-[U- ^{14}C]-phenylalanine (specific activity 350 mCi/mM) were obtained from New England Nuclear. Ninhydrin was obtained from the Sigma Chemical Corporation and L-leucine, actinomycin-D and 8-azaguanine from the Nutritional Biochemicals Corporation. Scintillation materials such as 2,5-diphenyl oxazole (PPO) and 1,4-bis-2-(5-phenyl-oxazolyl)-benzene were from Packard Instrument Company.

METHODS

Tissue Explants and Incubation Procedures: Fertilized chicken eggs incubated for 6-9 days in a commercial hatchery were obtained and placed immediately in a humidified incubator where they were maintained at 38°C and 80% humidity until they were ready for use. All incubations were made with long bone rudiments (tibiae) from 10-day old embryos carefully explanted by microdissection under culture medium. These tissues consist almost entirely of immature cartilage and they rapidly incorporate labelled proline into collagen in vitro. A large

part of the total protein output of these tissues is collagen. One tibia from each pair was used in the control, while the counter-member served as the test tissue. In most experiments, incubations were made with four tibiae in each vial. Tibias from these embryonic eggs, carefully dissected free of all adhering soft tissue, were used in these studies and both members of each pair were discarded if either was damaged during dissection. All incubations were made in a medium that contained a phosphate buffer, inorganic salts, and glucose (Krebs medium A) (72) at 37°C.

In experiments using the metabolic inhibitors 8-azaguanine (AZA) and actinomycin-D (Am-D), 62.5 ug/ml of the inhibitor was added along with the Poly IC to the incubation media at Time 0. All additives were dissolved in the medium and all incubations were made in a final volume of 2.5 ml, usually in screw-capped vials such as those used for scintillation counting; the vials were placed in a shaker-waterbath maintained at 37°C.

In all cases, the tissues were pre-incubated for fifteen minutes in an experimental medium before the radioactive precursor was added. All incubations were terminated by homogenizing the tissues in chilled distilled water. The homogenates were then dialyzed against water for 20 hr. The samples to be assayed for radioactive amino acid incorporation were hydrolyzed with 6.0 N HCl for 21 hours at 110° C in sealed test tubes. The

hydrolyzates were evaporated to dryness, in vacuo and the residues dissolved in 4 ml distilled water and appropriate aliquots were assayed for determination of total radioactivity incorporated, radioactive hydroxyproline and total protein.

Assay Procedures

Radioactivity: Radioactivity was determined in a liquid scintillation counter (Packard Tri-Carb, Model 3320) with the scintillation mixture described by Prockop and Ebert (73). For this 0.1 to 0.2 ml aliquots were placed in counting vials and 15.0 ml of scintillation fluid added and the samples were then counted. Observed counts per minute (cpm) were corrected to disintegrations per minute (dpm) after quench correction by the use of the automatic external standard or by adding internal standards. The scintillation counter had a background of 20 counts per minute.

Total Protein: Total protein content in the hydrolyzate was determined by assaying for total ninhydrin reactive material by the Rosen modification (74) of the Yemm and Cocking procedure (75) using leucine standards. Concentrations were computed in terms of umole of leucine equivalents. Specific activity, expressed as Dpm/umol leucine equivalent, was used as a measure of protein synthesis.

Radioactive Hydroxyproline: Assays for radioactive

hydroxyproline were made by the procedure described by Juva and Prockop (76). In some experiments, the incorporation of labelled proline into collagenous sequence was confirmed by the use of the most purified collagenase preparation available at this time. For this assay, the initial homogenates of labelled tissues were dialyzed in the cold against several changes of 50 mM TRIS-HCl, pH 7.6, containing 1 mM CaCl_2 . After dialysis, collagenase was introduced into the dialysis bag to a final concentration of 100 ug/ml, in a final volume of 5.0 ml and the dialysis bags were resealed and placed in screw-capped tubes containing 20 ml of the buffer. The tubes were capped, placed in a 37°C incubator and the contents including the dialysis bag were gently stirred for 6-24 hr. After incubation, the contents of the bag and the dialyzates were analyzed for radioactivity and the results were corrected for the presence of dialyzable materials inside the bag. The contents of the dialysis bag represented non-collagenous proteins while the dialyzates represented label incorporated into the collagenous sequence.

RESULTS

It is known that 10-day old chick embryo tibiae actively incorporate labeled proline into non-dialyzable peptide fractions. Proline and hydroxyproline account for approximately one-fourth of the total amino acid content in collagen and hydroxyproline is formed exclusively from proline already incorporated into peptide linkage. For this reason it is of advantage to use radioactive proline as a marker for protein synthesis in tissues actively engaged in the synthesis of collagen. Treatment of the proline and hydroxyproline labeled protein from this tissue with collagenase renders up to two-thirds of the label into a dialyzable form, suggesting that the label is present in collagenous sequence (77). Assays for labeled hydroxyproline support the collagenase data and indicate that collagen, containing about one-fourth proline and hydroxyproline residues accounts for approximately two-thirds of the total protein synthesis in vitro in this system (78).

Results to be discussed later in this report showed a higher rate of conversion of radioactive proline to hydroxyproline in the presence of Poly IC, suggesting a specific stimulation of collagen synthesis by the tissue. Also, in experiments to be reported here we also studied the incorporation of labeled phenylalanine, an amino acid

present only in trace amounts in collagen. These experiments did not show a similar increase in incorporation of phenylalanine into non-dialyzable fractions in the presence of Poly IC, confirming the suggestion that the Poly IC-stimulated protein synthesis in this tissue is largely collagen.

The specific nature of the incorporation of proline into collagenous sequence was confirmed through the use of the most purified collagenase preparations available at this time (Table I). As seen in Table I, using 2 tibiae/vial, concentrations of Poly IC in the range of 10 ug/ml to 1000 ug/ml caused a significant ($p < 0.01$) stimulation of proline incorporation into the collagenous sequence. Control tissues incorporated between 54% and 56% of the total radioactive proline uptake into collagenase digestible material. Addition of Poly IC increased the incorporation of proline into the collagenous sequence, suggesting that Poly IC did indeed cause specific stimulation of collagen synthesis. Based on these results, a concentration of 100 ug/ml Poly IC was used in all subsequent experiments.

Additional information concerning the specific nature of the stimulation of collagen by Poly IC was derived from experiments where an increased conversion of proline to hydroxyproline was observed. Hydroxyproline accounts for approximately 42% of the total imino acid residues in this species of collagen. Since radio-

TABLE I

EFFECT OF POLY IC ON THE SYNTHESIS OF COLLAGENASE-HYDROLYZABLE MATERIAL

Conc Poly IC ug/ml	Total ³ H-proline Radioactivity Incorporated DPM x 10 ⁻³				Radioactivity Dialyzed Out DPM x 10 ⁻³				Radioactivity Inside Bag DPM x 10 ⁻³			
	1	2	3	4	Mean S.D.	t	1	2	3	4	Mean S.D.	t
0	10.61	9.36	8.74	8.20	9.23 ± 0.52		5.75	5.24	4.80	4.35	5.04 ± 0.90	
10	12.47	11.96	12.85	11.72	12.25 ± 0.24 **		8.24	7.18	7.84	7.15	7.60 ± 0.27 *	
0	10.26	8.90	8.69	8.42	9.07 ± 0.41		5.40	4.82	4.70	4.55	4.86 ± 0.19	
100	13.65	14.72	14.96	15.20	14.63 ± 0.34 **		8.53	9.13	9.42	9.58	9.16 ± 0.23 **	
0	10.67	9.23	8.62	8.47	9.25 ± 0.50		5.80	5.08	4.65	4.58	5.03 ± 0.28	
500	13.40	13.27	12.60	12.93	13.05 ± 0.18 **		8.31	8.22	7.94	8.15	8.16 ± 0.80 **	
0	10.80	8.56	8.71	8.32	9.10 ± 0.57		6.00	4.71	4.75	4.49	4.99 ± 0.34	
1000	14.20	13.96	12.72	12.86	13.44 ± 0.32 **		8.77	8.79	7.89	7.85	8.32 ± 0.26 **	
							4.86	4.12	3.93	3.85	4.19 ± 0.23	
							4.24	4.78	5.01	4.57	4.65 ± 0.16 N/S	
							4.86	4.10	3.99	3.88	4.21 ± 0.22	
							5.12	5.59	5.54	5.62	5.47 ± 0.12 **	
							4.81	4.15	3.97	3.90	4.21 ± 0.28	
							5.05	5.04	4.66	4.79	4.89 ± 0.10 *	
							4.83	3.85	3.96	3.83	4.12 ± 0.24	
							5.35	5.17	4.83	5.02	5.09 ± 0.11 *	

* = p < 0.05 t test

** = p < 0.01 t test

N/S = not significant

Incubations, collagenase treatment and subsequent assays were performed as described in text. Each set of tissues (2 tibiae) was incubated for 1 hour with 2 uc L-[3,4-³H]-proline plus various concentrations of Poly IC.

active hydroxyproline is formed only from radioactive proline during collagen synthesis and since hydroxyproline occurs uniquely in collagen, one may calculate the proportionate contributions of collagen synthesis and non-collagenous protein synthesis from the amount of radioactive hydroxyproline synthesized. Thus, under conditions where all possible proline residues are hydroxylated,

$$\frac{\text{Radioactivity in Collagen}}{\text{Radioactivity in Hydroxyproline}} = \frac{100}{42} \times$$

and,

$$\frac{\text{Radioactivity in non-collagenous proteins}}{\text{Total radioactivity incorporated}} = \frac{\text{Radioactivity in collagen}}{\text{Total radioactivity incorporated}} -$$

Table II shows the proportions of collagen and non-collagenous proteins synthesized in the presence of Poly IC. Increased collagen synthesis accounted for almost all of the excess proline incorporated in the presence of Poly IC, whereas, there was a slight ($p < 0.05$) depression in the synthesis of non-collagenous protein at 30 minutes. In later experiments with phenylalanine, radioactivity in non-collagenous proteins was consistently lower than that of control value. These observations suggested that the incorporation of proline

EFFECT OF POLY IC ON COLLAGEN BIOSYNTHESIS

* = $p < 0.05$ t test
 ** = $p < 0.01$ t test
 N/S = not significant

15.

alone may be used as an index of collagen synthesis and of total protein synthesis. In several of the experiments presented here, collagen synthesis is denoted simply in terms of specific activity of the proline labelled protein synthesized, since the uptake of proline into peptides represents mainly collagen synthesis in this system. Thus these data are expressed in terms of dpm/ μ m amino nitrogen (total ninhydrin reactive material in the protein hydrolysate) compared with leucine standards and expressed as leucine equivalents (L.E.).

The time course of proline incorporation in the presence of Poly IC was examined. Tissues (2 tibiae/vial) were incubated with Poly IC (100 μ g/ml) for varying lengths of time. As shown in Figure 1, embryonic chick cartilage treated with Poly IC showed a significant increase (Table III) in incorporation of label over control after only 30 minutes ($p < 0.01$).

Experiments using radioactive phenylalanine incorporation, instead of radioactive proline incorporation, into the non-dialyzable fraction of chicken embryo cartilage were performed to confirm the previous experiments that the stimulation was largely restricted to collagen. Collagen, uniquely high in the imino acids proline and hydroxyproline has very few phenylalanine residues in its polypeptide chain. Since phenylalanine residues occur more frequently in other protein constituents of the cells, an increase in incor-

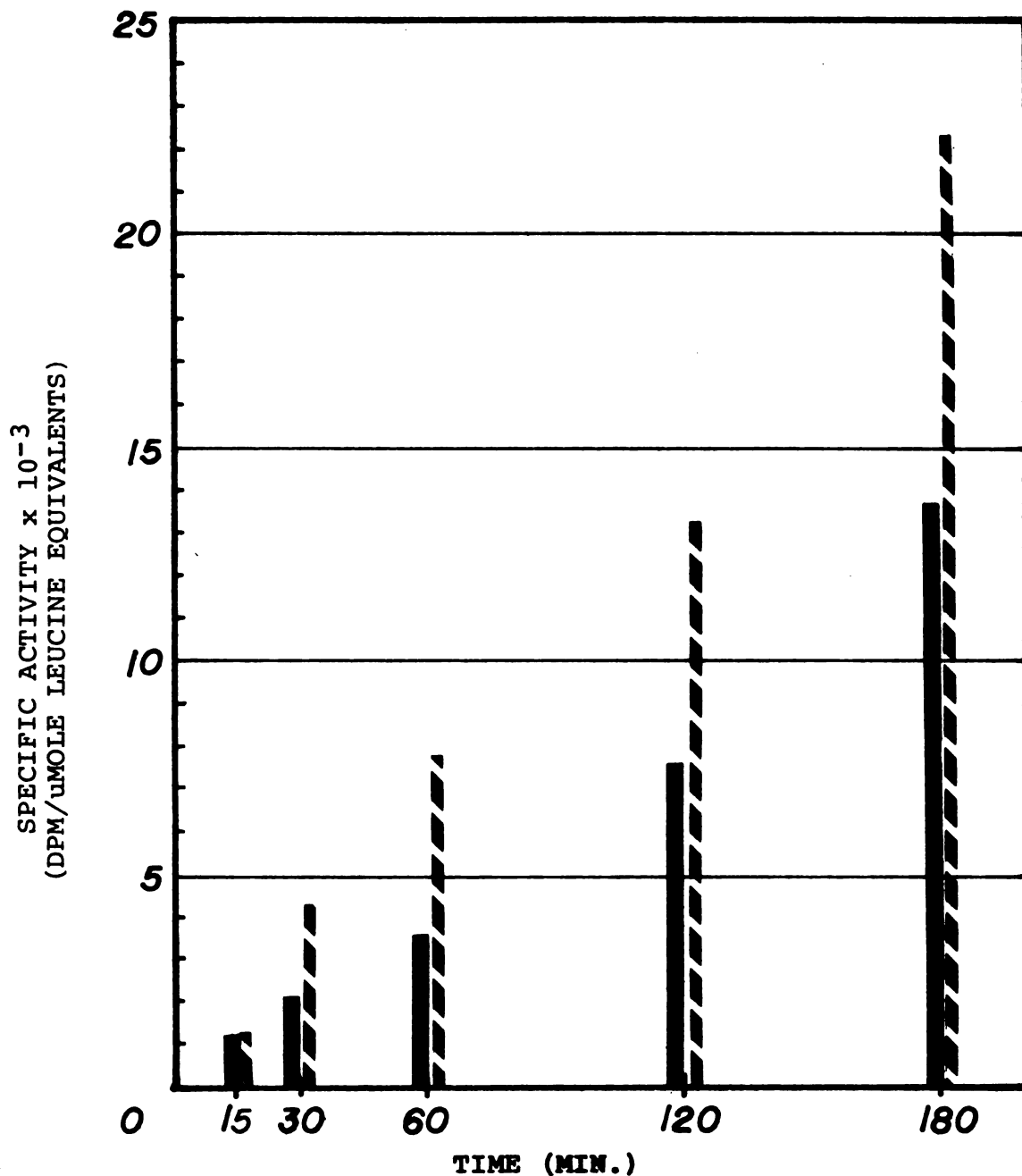


FIGURE 1

TIME COURSE OF THE STIMULATORY EFFECT
OF PGLY IC ON PROLINE INCORPORATION IN CARTILAGE

Incorporation of L-[3,4³H]-proline into chick embryo cartilage continuously labelled under control condition. ▨ Specific Activity of tissues treated with 100 ug of Poly IC. ■ Specific Activity of Controls.

TABLE III

STATISTICAL ANALYSIS OF FIGURE 1

Time	Sample	1	2	3	4	5	6	7	Mean	S.D.	Test
Min.											
15	Control	-	1.14	1.01	1.33	1.27	1.35	-	1.22	±0.06	N/S
	Poly IC	-	1.07	1.21	1.32	1.42	1.21	-	1.25	±0.06	
30	Control	1.80	1.96	2.01	1.82	2.40	2.30	2.36	2.09	±0.10	**
	Poly IC	3.90	4.19	4.23	4.62	4.15	4.58	3.92	4.23	±0.11	
60	Control	3.60	4.10	3.82	4.32	2.15	3.03	3.96	3.57	±0.28	**
	Poly IC	7.80	6.00	7.22	8.41	8.00	8.10	8.40	7.70	±0.32	
120	Control	7.00	7.75	7.96	6.93	7.75	7.20	8.23	7.55	±0.19	**
	Poly IC	12.96	10.87	12.12	13.78	13.00	14.21	16.31	13.31	±0.65	
180	Control	12.10	11.29	11.97	13.24	14.80	-	12.34	12.62	±0.51	**
	Poly IC	19.00	23.23	21.62	22.61	21.00	-	26.10	22.26	±0.97	

- = not tested
 ** = $p < 0.01$ t test
 N/S = not significant

poration of this amino acid into the non-dialyzable portion of four chick embryonic tibia in the presence of Poly IC would indicate that non-collagenous proteins were also stimulated; however, as shown in Figure 2, Poly IC did not stimulate the incorporation of radioactive phenylalanine. Rather, in the presence of Poly IC, radioactive incorporation into embryonic cartilage was significantly less ($p < 0.05$) than in controls (Table IV), suggesting that perhaps Poly IC depressed the relative rates of synthesis of proteins containing phenylalanine while stimulating collagen synthesis.

In order to explain the mechanism of the stimulatory effect of Poly IC on collagen biosynthesis, experiments with metabolic inhibitors were used. Actinomycin-D and 8-azaguanine which inhibit RNA synthesis were used to determine if the stimulatory effect of Poly IC on collagen biosynthesis involved increased synthesis of informational macromolecules. As shown in Figure 3, 8-azaguanine and actinomycin-D inhibited the stimulatory effect of Poly IC on collagen biosynthesis, suggesting that Poly IC was affecting protein synthesis at the transcriptional level. Statistical analysis (Table V) demonstrates a significant difference at the $p < 0.01$ level. These metabolic inhibitors markedly depressed the incorporation of proline into the non-dialyzable fraction of chick embryonic tibiae (4 tibiae/vial).

Incorporation of radioactive uridine into the non-

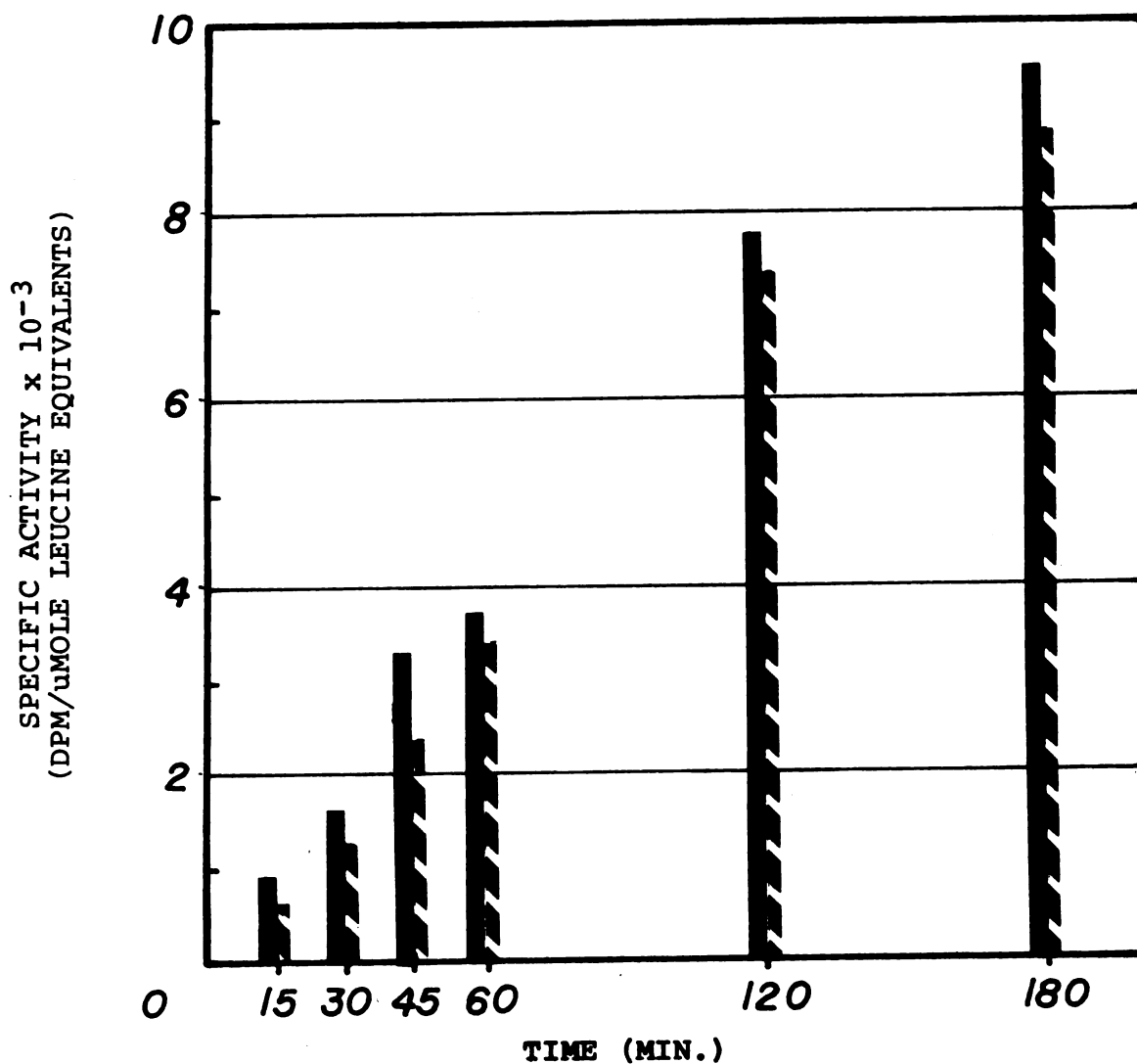


FIGURE 2

THE EFFECT OF POLY IC ON
PHENYLALANINE INCORPORATION INTO CARTILAGE

Incorporation of L-[U-¹⁴C]-phenylalanine into the non-dialyzable fraction of embryonic cartilage continuously labelled with the isotope. Specific Activity of the samples with Poly IC ▨▨▨▨. Specific Activity of the control samples with no Poly IC ■■■■.

TABLE IV

STATISTICAL ANALYSIS OF FIGURE 2

Time Min.	Sample	Specific Activity $\times 10^{-3}$			Mean	S.D.	Test
		1	2	3			
15	Control Poly IC	0.88 0.66	- -	0.95 0.59	0.92 0.63	± 0.04 ± 0.04	N/S
30	Control Poly IC	1.80 1.37	1.52 1.22	1.61 1.31	1.64 1.30	± 0.08 ± 0.04	*
45	Control Poly IC	- -	3.30 2.44	3.40 2.36	3.35 2.40	± 0.05 ± 0.04	*
60	Control Poly IC	3.70 3.46	3.62 3.50	3.82 3.42	3.71 3.46	± 0.06 ± 0.02	*
120	Control Poly IC	7.60 7.40	7.86 7.24	7.81 7.31	7.76 7.32	± 0.11 ± 0.04	*
180	Control Poly IC	9.84 8.85	9.34 8.90	9.55 8.70	9.58 8.82	± 0.15 ± 0.06	**

- = not tested
 * = $p < 0.05$ t test
 ** = $p < 0.01$ t test
 N/S = not significant

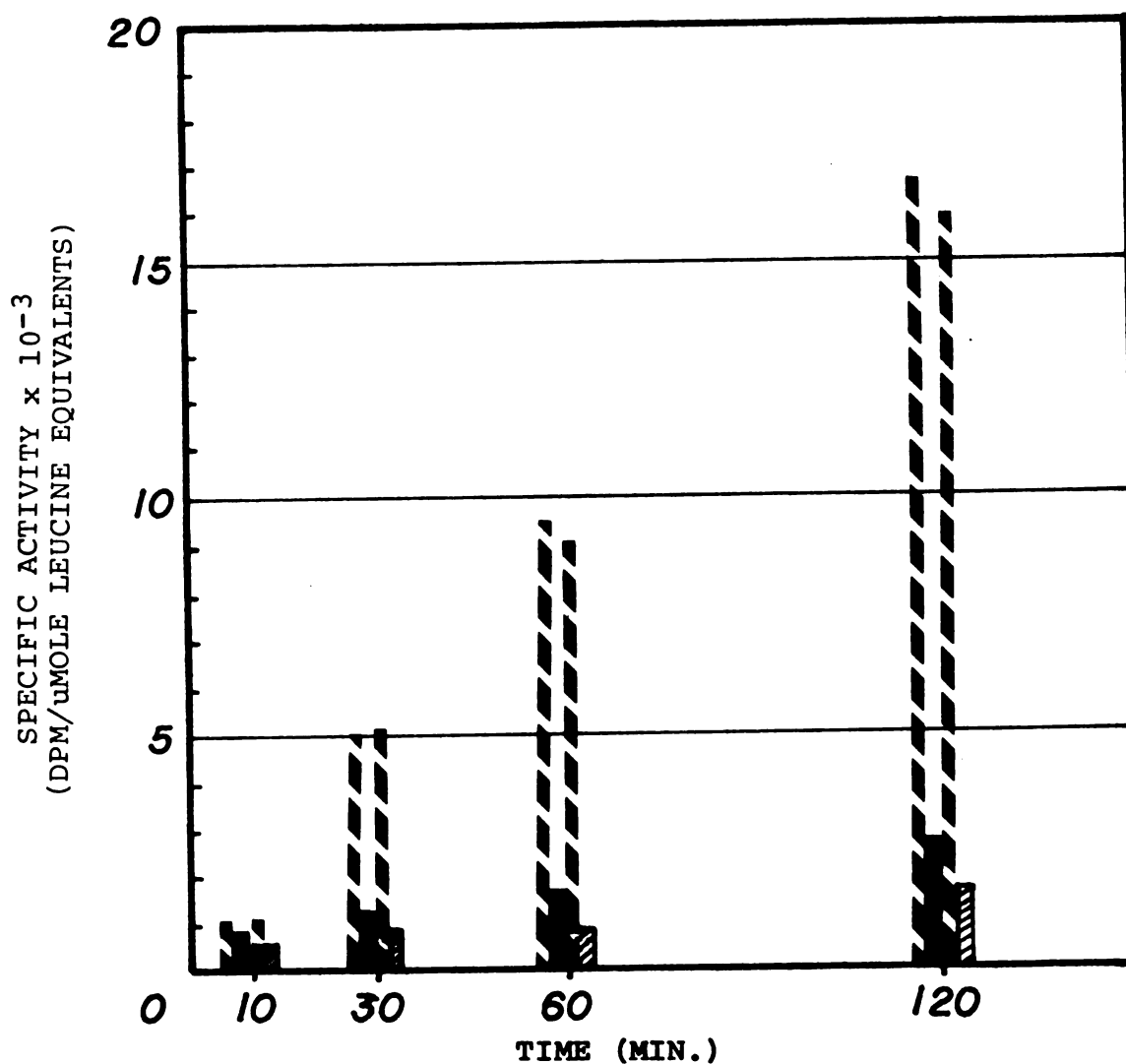


FIGURE 3

THE EFFECT OF POLY IC ON PROLINE INCORPORATION
IN THE PRESENCE OF METABOLIC INHIBITORS

Incorporation of L-[3,4³H]-proline into the non-dialyzable portion of embryonic cartilage continuously labelled with the isotope. Specific Activity of samples with Poly IC ▨▨. Specific Activity of samples with 8-azaguanine plus Poly IC ■■■. Specific Activity of samples with actinomycin-D plus Poly IC ▩▩▩.

TABLE V

STATISTICAL ANALYSIS OF FIGURE 3

Time	Sample	Specific Activity x 10 ⁻³							Mean	S.D.	Test
Min.		1	2	3	4	5	6	7	8		
15	Poly IC	1.27	1.03	1.42	1.37	1.12	1.23	1.02	0.82	1.16	±0.07
	Poly IC+AZA	0.63	0.97	1.44	1.03	0.84	1.52	0.51	0.37	0.91	±0.15
	Poly IC	1.08	1.21	1.16	1.14	-	-	1.26	0.97	1.14	±0.42
	Poly IC+Am-D	0.31	0.46	0.97	0.80	-	-	0.42	0.78	0.63	±0.11
30	Poly IC	5.78	4.92	4.82	5.16	5.05	4.50	5.38	-	5.09	±0.16
	Poly IC+AZA	0.82	1.25	0.96	1.53	2.12	1.00	1.06	-	1.26	±0.18
	Poly IC	5.27	3.95	4.97	6.35	-	5.31	-	-	5.17	±0.38
	Poly IC+Am-D	0.51	0.62	0.94	1.58	-	1.15	-	-	0.96	±0.19
60	Poly IC	10.46	8.76	8.10	9.94	10.67	-	-	-	9.59	±0.49
	Poly IC+AZA	0.90	1.31	1.25	2.10	2.82	-	-	-	1.68	±0.35
	Poly IC	9.23	8.25	8.60	10.16	10.00	8.00	8.00	9.80	9.15	±0.33
	Poly IC+Am-D	0.88	0.96	9.52	1.14	0.75	0.80	1.00	-	0.87	±0.08
120	Poly IC	-	-	-	-	18.40	15.60	16.20	-	16.73	±0.85
	Poly IC+AZA	-	-	-	-	2.10	2.96	2.81	-	2.63	±0.27
	Poly IC	-	-	-	-	18.75	14.80	15.72	14.38	15.91	±0.99
	Poly IC+Am-D	-	-	-	-	0.79	1.62	1.92	2.11	1.62	±0.30

- = not tested
 ** = p 0.01 t test
 N/S = not significant

AZA = 8-azaguanine
 Am-D = actinomycin-D

dialyzable portion of chick embryonic tibia is a good index of the mRNA synthesis in the tissue. Although uridine also appears in ribosomal RNA and transfer RNA within the cell, the turnover rate of these two species of RNA is slow and therefore, the short-term incorporation of radioactive uridine into these components may be considered negligible in comparison to mRNA. As shown in Figure 4, incorporation of tritiated uridine into non-dialyzable fractions of four 10-day old chick embryo cartilage explants in the presence of 100 ug/ml of Poly IC was markedly enhanced. Figure 4 illustrates continuous labelling with uridine over time. Statistical evaluation (Table VI) indicates a significant increase at the $p < 0.01$ level. Subsequent experiments using pulse-labelling of tritiated uridine confirm the increased incorporation of this substrate in the presence of Poly IC.

In other experiments, actinomycin-D in the presence of Poly IC depressed uridine incorporation very markedly. These results are consistent with the current viewpoint that actinomycin-D acts primarily to inhibit the DNA-dependent synthesis of RNA, including mRNA, and as a consequence of breakdown of preexisting mRNA, a secondary inhibition of protein synthesis occurs.

It was of interest to know how long the stimulatory effect of Poly IC in collagen biosynthesis would last if the synthetic double stranded polynucleotide was removed from the incubation media. Experiments were conducted

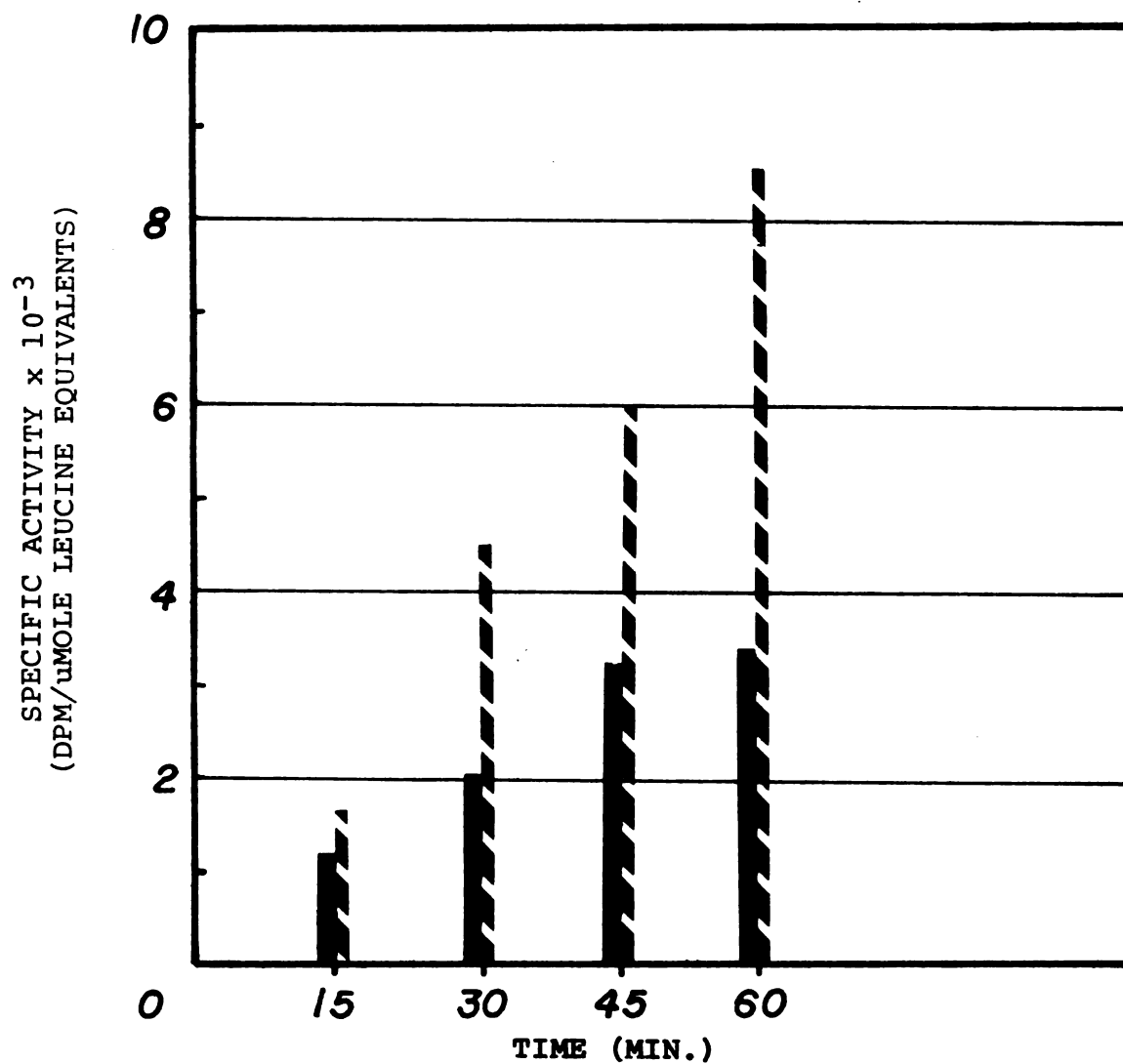


FIGURE 4

THE EFFECT OF POLY IC ON URIDINE INCORPORATION

Incorporation of $[5-^3\text{H}]$ -uridine into embryonic cartilage continuously labelled with the isotope. Specific Activity of samples with Poly IC . Specific Activity of control samples with no Poly IC .

TABLE VI

STATISTICAL ANALYSIS OF FIGURE 4

Time Min.	Sample	Specific Activity x 10 ⁻³						Mean	S.D.	Test
		1	2	3	4	5	6			
15	Control Poly IC	1.33	1.46	0.88	0.92	1.36	0.97	1.15	±0.11	*
		1.58	2.00	1.34	1.43	1.96	1.28	1.60	±0.13	
30	Control Poly IC	2.41	1.62	2.10	2.02	2.10	1.70	1.99	±0.12	**
		4.20	5.26	4.00	4.25	4.56	4.42	4.45	±0.18	
45	Control Poly IC	3.40	2.96	3.34	2.96	3.32	3.21	3.20	±0.08	**
		6.15	5.60	5.84	5.92	5.90	5.97	5.90	±0.07	
60	Control Poly IC	3.68	3.20	3.02	3.50	3.15	3.60	3.36	±0.11	**
		8.25	8.60	8.10	9.32	8.00	8.60	8.48	±0.19	

* = p 0.05 t test

** = p 0.01 t test

incubating 10-day old chick embryo cartilage (4 tibiae/vial) explants with Poly IC (100 ug/ml) for 30 minutes, then washing these tissues with 2.5 ml Krebs IIA media and placing these explants into fresh media plus specifically labelled proline, continuously incubating them in the presence of the isotope for various times.

As shown in Figure 5 and Table VII, the incorporation of this labelled imino acid treated previously for 60 minutes with Poly IC significantly ($p < 0.01$) stimulated collagen biosynthesis for at least 45 minutes. After 120 minutes no significant difference in the incorporation of radioisotope, in the samples previously treated with Poly IC, was apparent.

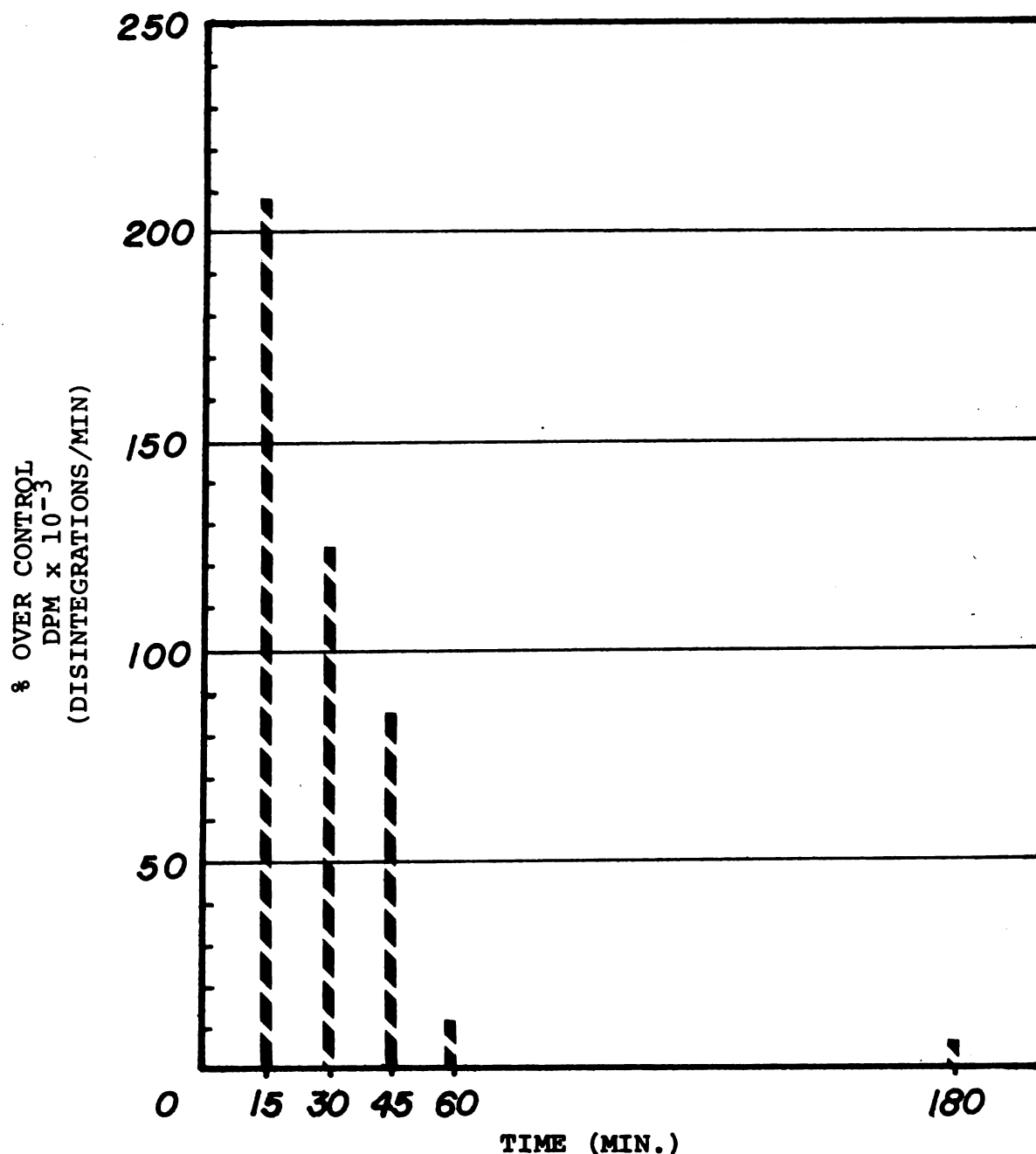


FIGURE 5

TIME COURSE IN DISAPPEARANCE OF THE STIMULATORY
EFFECT OF POLY IC, AFTER POLY IC IS REMOVED
FROM THE MEDIUM

Incorporation of L-[3,4³H]-proline into the non-dialyzable fraction of chick cartilage continuously labelled with the amino acid after removal of Poly IC after 1 hour incubation from the incubation media. $\blacktriangle$ % stimulation of incorporation of isotope over control after removal of Poly IC from the media.

TABLE VII

STATISTICAL ANALYSIS OF FIGURE 5

Time Min.	Sample	1	2	3	4	5	Mean	S.D.	Test
DPM x 10 ⁻³ (Disintegrations/Min.)									
15	Control	8.58	3.02	4.01	6.01	4.50	5.24	±0.97	
	Poly IC	23.50	14.06	15.00	14.20	14.20	16.39	±1.79	**
30	Control	12.18	8.55	9.34	9.72	9.13	9.79	±0.63	
	Poly IC	25.92	18.82	21.20	23.60	22.11	22.33	±1.19	**
45	Control	20.00	12.60	14.00	15.20	14.21	15.20	±1.27	
	Poly IC	36.71	38.80	24.00	26.00	24.20	28.34	±2.42	**
60	Control	35.10	18.00	27.00	28.00	29.62	27.54	±2.77	
	Poly IC	39.30	22.00	30.00	34.00	31.25	31.30	±2.83	N/S
180	Control	52.05	49.22	54.60	47.32	51.63	51.06	±1.25	
	Poly IC	56.00	50.21	56.84	52.36	57.21	54.52	±1.37	N/S

** = p < 0.01 t test
N/S = not significant

DISCUSSION

Our studies indicate a significant effect of Poly IC on cellular metabolism in embryonic cartilage which results in a marked enhancement of collagen synthesis. It was important in the initial stages of these studies to firmly establish the specific nature of the stimulation of collagen biosynthesis. As seen in Table II, most of the excess proline radioactivity incorporated in the presence of Poly IC appeared in collagen. These experiments were slightly complicated by the observation that the levels of hydroxylation of incorporated proline varied with the length of incubation time and the maximal hydroxyproline content did not always coincide with the point of greatest stimulation of proline incorporation. However, even if the data reported in Table II represented an experiment where the hydroxylation of all available proline residues was not complete, these data still emphasize the specificity of stimulation of collagen synthesis. The specific nature of stimulation was also confirmed (Table I) by the use of a bacterial collagenase preparation. It is also supported by our observation that the incorporation of phenylalanine, an amino acid present only in very small amounts in collagen, is not altered in the same manner as proline. The observation that the incorporation of phenylalanine is slightly suppressed suggests that the

synthesis of noncollagenous proteins may be reduced in the presence of Poly IC. The ease and sensitivity with which the synthesis of collagen may be measured make it an ideal system for studying the regulation of protein synthesis in general.

The mechanism of alteration of protein synthesis in cartilage by Poly IC is not fully clear. It is unlikely that Poly IC increases protein synthesis by altering the permeability of the cells of the tissue. If this were the mode of action, the incorporation of proline and phenylalanine would be affected in the same manner. However, as noted earlier, proline incorporation is increased and phenylalanine incorporation falls below control levels. The reversal of the stimulatory effect in the presence of actinomycin-D and azaguanine suggests that permeability may not be the prime factor in enhancing collagen synthesis. Our experiments also indicate that the stimulatory effect of Poly IC is not apparent until after 15 minutes or longer following Poly IC administration. The stimulatory effect continues for at least 45 minutes after the stimulating polyribonucleotides have been removed from the medium, further eliminating a significant role for altered cellular permeability in the stimulation of collagen biosynthesis.

There is evidence that Poly IC may enter cells in culture (81-85). There are other investigators who indicate that Poly IC is adsorbed to the cell surface and

the strands separated; Poly C is then disrupted while part of Poly I enters into the cells (86,87).

In cartilage explants used by us, the cells are embedded in a mass of matrix consisting of collagen and sulfated protein polysaccharides. In the absence of suitable data, it is difficult to speculate whether Poly IC enters cells after partial degradation, and to what extent these observations may relate to our studies. In order for Poly IC to induce such marked changes in protein synthesis as observed by us, it may be safe to assume that at least a part of the effect of Poly IC on cartilage occurs after the entry of the synthetic double stranded polynucleotide into cells and its interaction with some regulatory molecules in the cytoplasm or in the nucleus.

Among these possibilities, one may include (a) competition for cytoplasmic RNase, resulting in a lowered degradation of cellular RNA, species predominant among which would be mRNAs; (b) interaction with a cytoplasmic repressor for collagen; and (c) induction of enhanced mRNA synthesis by unmasking certain specific regions of the genome, specifically, those concerned with the synthesis of collagen mRNA.

It is possible that Poly IC may compete for RNase in the cytoplasm (88). However, its stimulatory effect is eliminated by simultaneous administration of inhibitors of RNA synthesis. This would indicate that synthe-

sis of new RNA rather than degradation of preexisting RNA may be a more important regulatory mechanism in this case. The observation that the incorporation of phenylalanine is depressed also does not support a significant role for Poly IC-RNase interaction since all mRNA's should be equally protected rather than a specific one being protected while others are degraded.

An attractive possibility is that Poly IC interacts with a cytoplasmic repressor for collagen, in competition with the collagen mRNA. Collagen mRNA should be rich in cytidyl residues since one-fourth of the total amino acid residues in collagen are proline and hydroxyproline, coded by CCC, CCU, CCA and CCG (89,90). Poly IC might have affinity for a repressor molecule which would bind a cytidine-rich RNA molecule. The existence of cytoplasmic repressors have been suggested by Tomkins and colleagues (91). They suggest that superinduction of tyrosine amino transferase in HTC cells in culture by inhibitors of RNA synthesis may be caused by their interaction with cytoplasmic repressors, thus making more mRNA available for translation. Several other investigators have presented evidence suggesting that newly synthesized mRNA may occur in association with a protein (92,93). It has been postulated that the protein may reversibly "mask" the mRNA and prevent its translation. This protein may be involved in protecting the mRNA for prolonged periods, pending the complete preparation of the cellular

machinery for the synthesis of proteins (94). Several authors have attributed a role in translation level controls to this protein (92,93,95-98). If the "masking" protein serves as a translation level repressor in a normally operating system, there may exist mechanisms for removal of this repressor or for otherwise "unmasking" the mRNA. However, the fact that actinomycin-D and 8-azaguanine markedly inhibit the stimulatory effect thus would make the putative cytoplasmic repressor appear to be only a minor factor in enhancing collagen synthesis.

Another interesting possibility is that Poly IC may unmask specific regions of the genome and cause increased transcription of collagen mRNA. Nuclear RNA synthesis is, at least in a general way, regulated by histones and investigators (99-101) have demonstrated that removal of histones results in increased RNA synthesis. Fenster (102) has related the amount of histone associated with DNA with the rate of synthesis of RNA. Poly IC, a polyanion, may interact with polycationic histones and stimulate transcription in the nucleus. Increased incorporation of uridine reflects such an increase in RNA synthesis, as observed in the present experiments; and a time lag of increase in incorporation of labelled uridine would allow the polyanion Poly IC to reach the nucleus and exert its effect. Additional specific regulatory mechanisms would have to be invoked to account for the specificity for collagen, however.

In the present experiments, an increase in labelled uridine incorporation was observed in chick embryo cartilage treated with Poly IC. Also, the observed time lag of 15 minutes in experiments using radioactive proline incorporation into the non-dialyzable fractions of the cartilage explants would allow the polyanion synthetic double stranded polynucleotide to reach the nucleus and exert its effect on the histone-DNA complex and on the synthesis of mRNA. Our uridine and azaguanine experiments suggest that Poly IC is increasing collagen synthesis by affecting transcriptional level controls.

SUMMARY AND CONCLUSIONS

The synthetic double stranded RNA poly-inosinic poly-cytidylic acid (Poly IC) markedly alters the synthesis of proteins in cartilage, and thus provides a useful tool for studying the regulation of protein synthesis. Two-thirds of proline incorporated in 10-day old chick embryo cartilage is accounted for by collagen, and in these studies the mechanism of Poly IC-induced stimulation of protein synthesis in cartilage was investigated to obtain some information concerning the regulation of collagen biosynthesis. Poly IC markedly stimulated the incorporation of proline into non-dialyzable fractions in cartilage explants. Assays based on hydroxyproline measurements and the use of a collagenase preparation demonstrated that most of the excess radioactivity incorporated in the presence of Poly IC was in collagenous sequences. The incorporation of phenylalanine, an amino acid present only in trace amounts in collagen was somewhat suppressed. The stimulatory effect of Poly IC was maximal at 20 to 30 minutes after administration. Increased incorporation of ^3H -uridine suggested increased RNA synthesis. The role of induced mRNA synthesis was also suggested by the observation that inhibitors of RNA synthesis abolished the stimulatory effect on proline incorporation. These observations suggest that in

developing mesenchymal systems the regulation of protein synthesis may be related to the presence of macromolecular polyelectrolytes.

BIBLIOGRAPHY

1. Grant, M.E. and Prockop, D.J., New England J. of Med., 286, 194 (1972).
2. Grant, M.E. and Prockop, D.J., New England J. of Med., 286, 242 (1972).
3. Grant, M.E. and Prockop, D.J., New England J. of Med., 286, 291 (1972).
4. Peterkofsky, B. and Udenfriend, S., J. Biol. Chem., 238, 2966 (1963).
5. Prockop, D.J. and Juva, K., Proc. Nat. Acad. Sci., 53, 661 (1965).
6. Lukens, L.N., J. Biol. Chem., 240, 1661 (1965).
7. Udenfriend, S., Science, 152, 1335 (1966).
8. Bhatnagar, R.S., Rosenbloom, J., Kirvirikko, K.I. and Prockop, D.J., Biochem. Biosphys. Acta, 149, 273 (1967).
9. Rosenbloom, J., Bhatnagar, R.S. and Prockop, D.J., Biochem. Biophys. Acta, 149, 259 (1967).
10. Bhatnagar, R.S., Prockop, D.J. and Rosenbloom, J., Science, 158, 492 (1967).
11. Prockop, D.J., Chemistry and Molecular Biology of the Intercellular Matrix, Academic Press, London and New York, 1970, Vol. I, pp. 335-370.
12. Miller, R.L. and Udenfriend, S., Arch. Biochem. Biophys., 139, 104 (1970).
13. Lazarides, E. and Lukens, L.N., Nature, 232, 37 (1971).
14. Udenfriend, S., Chemistry and Molecular Biology of the Intercellular Matrix, Academic Press, London and New York, 1970, Vol. I, pp. 371-384.
15. Juva, K. and Prockop, D.J., J. Biol. Chem., 241, 4419 (1966).
16. Juva, K., Prockop, D.J. and Cooper, G.W., Science, 152, 95 (1966).

17. Hutton, J.J., Marglin, A., Witkop, B. et al,
Arch. Biochem. Biophys., 125, 779 (1968).
18. Kivirikko, K.I. and Prockop, D.J., J. Biol. Chem.
242, 4007 (1967).
19. Bornstein, P., J. Biol. Chem. 242, 2572 (1967).
20. Butler, W.T., Science, 161, 769 (1968).
21. Hutton, J.J., Tappel, A.L. and Udenfriend, S.,
Arch. Biochem. Biophys., 118, 231 (1967).
22. Rhoads, R.E. and Udenfriend, S., Arch. Biochem.
Biophys. 139, 329 (1970).
23. Mussini, E., Hutton, J.J. and Udenfriend, S.,
Science, 157, 927 (1967).
25. Bosmann, H.B. and Eylar, E.H., Biochem. Biophys.
Res. Comm., 33, 340 (1968).
26. Blumenkrantz, N., Rosenbloom, J. and Prockop, D.J.,
Biochem. Biophys. Acta, 192, 81 (1969).
27. Morgan, P.H., Jacobs, H.G., Segrest, J.P. et al,
J. Biol. Chem. 245, 5042 (1970).
28. Eylar, E.H., J. Theoret. Biol., 10, 89 (1965).
29. Bhatnagar, R.S., Kivirikko, K.I., Rosenbloom, J.
and Prockop, D.J., Proc. Natl. Acad. Sci., 58,
248 (1967).
30. Bhatnagar, R.S., Rapaka, S.S.R., Nature New Biology,
237, 92 (1971).
31. Martin, D.W. and Tomkins, G.M., Proc. U.S. Nat.
Acad. Sci., 65, 1064 (1970).
32. Brieger, H. and Cross, P., Arch. Environ. Health,
13, 38 (1966).
33. Wexler, B.C., Judd, J.T., Kittinger, G.W., Athero-
scler. Res., 4, 397 (1964).
34. Field, A.K., Tytell, A.A., Lampson, G.P. and
Hillman, M.R., Proc. Nat. Acad. Sci. U.S., 58,
1004 (1967).
35. Lampson, G.P., Tytell, A.A., Field, A.K., Nemes, M.M.
and Hilleman, M.R., Proc. Nat. Acad. Sci. U.S.,
58, 782 (1967).

36. Tytell, A.A., Lampson, G.P., Field, A.K. and Hilleman, M.R., Proc. Nat. Acad. Sci. U.S., 58, 1719 (1967).
37. Field, A.K., Lampson, G.P., Tytell, A.A., Nemes, M.M. and Hilleman, M.R., Proc. Nat. Acad. Sci. U.S., 58, 2102 (1967).
38. Field, A.K., Tytell, A.A., Lampson, G.P. and Hilleman, M.R., Proc. Nat. Acad. Sci. U.S., 61, 340 (1968).
39. Hilleman, M.R., J. Cell Physiol., 71, 43 (1968).
40. Weinstein, M.J., Waitz, J.A., Came, P.E., Nature, 226, 1541 (1970).
41. Kreider, J.W., Fed. Proc. Abst., 29, 2104 (1970).
42. Larson, V.M., Pantiliakis, P.N. and Hilleman, M.R., Proc. Soc. Exp. Biol. Med., 133, 14 (1970).
43. Levy, H.B., U.S. Japan Seminar on Interferon, Tokyo, 1969.
44. Woodhour, A.F., Freidman, A., Tytell, A.A. and Hilleman, M.R., Proc. Soc. Exp. Biol. Med., 131, 809 (1969).
45. Winchurch, R. and Braun, W., Nature, 223, 718 (1969).
46. Braun, W. and Wakano, W., Science, 157, 819 (1967).
47. Turner, W., Chan, S.P. and Chirigos, M.A., Proc. Soc. Exp. Biol. Med., 133, 334 (1970).
48. Cantor, H., Asofsky, R. and Levy, H.B., J. Immunol., 104, 1035 (1970).
49. Baugh, C.L., Tytell, A.A. and Hilleman, M.R., Proc. Nat. Acad. Sci. U.S., 137, 1194 (1971).
50. Ho, M. and Enders, J.F., Virology, 9, 446 (1959).
51. Wagner, R.R., Virology, 13, 323 (1961).
52. Grossberg, S.E. and Holland, J.J., J. Immunol., 88, 708 (1962).
53. Sonnabend, J.A., and Friedman, R.M., Interferons, North-Holland Publ., Amsterdam, 1967, pp. 202-231.
54. Joklik, W.K. and Merigan, T.C., Proc. Nat. Acad. Sci. U.S., 56, 558 (1966).

55. Marcus, P.I. and Salb, J.M., *Virology*, 30, 502 (1966).
56. Vilcek, J., Rossman, T.G. and Varacalli, F., *Nature*, 223, 682 (1969).
57. Vilcek, J., *Ann N.Y. Acad. Sci.*, (1969).
58. Bausek, G.H. and Merigan, T.C., *Proc. Soc. Exp. Biol. Med.*, 133, 982 (1970).
59. Finkelstein, M.S., Bansek, G.H. and Merigan, T.C., *Science*, 161, 465 (1968).
60. Pakoff and Boroff, P., *Biochem. Biophys. Acta*, 174, 108 (1969).
61. Niu, M.C., *Develop. Biol.*, 7, 379 (1963).
62. Fishman, M., Hammerstrom, R.A. and Bond, V.P., *Nature*, 198, 549 (1963).
63. Bishop, D.C. and Abramoff, P., *Proc. Soc. Exp. Biol. Med.*, 120, 378 (1965).
64. Shanmugam, C. and Bhargava, P.M., *Biochem. J.*, 99, 297 (1966).
65. Ellem, K.A.O., and Colter, J.S., *Virology*, 15, 113 (1961).
66. Colter, J.S., *Proc. 5th Can Cancer Res. Conf.*, 1962, 5, 65.
67. Amos, H. and Kearns, K.E., *Exp. Cell Res.*, 32, 14 (1963).
68. Colby, C. and Chamberlin, M.J., *Proc. Nat. Acad. Sci. U.S.*, 63, 160 (1969).
69. Harel, L., Harel, J., Lacour, F., *C.R. Acad. Sci.*, 253, 2000 (1961).
70. Chin, P.H. and Silverman, M.S., *J. Immunol.*, 99, 476 (1967).
71. Levy, H.B., Riley, F., Margolis, S.A., Lou, L., and Robson, A., *Fed. Proc. Abstracts*, 29, 1361 (1969).
72. Krebs, H.A., *Biochem. Biophys. Acta*, 4, 249 (1950).
73. Prockop, J. and Ebert, P.S., *Anal. Biochem.*, 6, 263 (1963).
74. Rosen, H., *Arch. Biochem. Biophys.*, 67, 10 (1957).

75. Yemm, E.W. and Cocking, L.D., *The Analyst*, 80, 209 (1955).
76. Juva, K. and Prockop, D.J., *Anal. Biochem.*, 15, 77 (1966).
77. Bhatnagar, R.S., Kivirikko, K.I. and Prockop, D.J., *Biochem. Biophys. Acta*, 154, 196 (1968).
78. Bhatnagar, R.S. and Prockop, D.J., *Biochem. Biophys. Acta*, 130, 383 (1966).
79. Yoon, C.H., *Exp. Cell Res.*, 38, 386 (1965).
80. Levy, H.B., *Arch Intern. Med.*, 126, 78 (1970).
81. Bausek, G.H., and Merigan, T.C., *Virology*, 39, 491 (1969).
82. Colby, C. and Chamberlain, M.J., *Proc. Nat. Acad. Sci.*, 63, 160 (1969).
83. DeClercq, E., Wells, R.D. and Merigan, T.C., *Virology*, 47, 405 (1972).
84. Levy, H.B., Asotsky, R., Riley, R., Garapin, A., Cantor, H. and Adamson, R., *Ann. N. Y. Acad. Sci.*, 173, 640 (1970).
85. Prose, P.H., Friedman-Kein, A. and Vilcek, J., *J. Gen. Physiol.*, 56, 99 (1970).
86. Schell, P.L., *Biochemica et Biophysica Acta*, 240, 472 (1971).
87. Kelly, T.A., Levy, H.B., *Proc. Nat. Acad. Sci.*, 144, 534, (1973).
88. Buchler, C.E., *Fed. Proc. Abstracts*, 29, 1356 (1970).
89. Khorana, H.G., *Fed. Proc.*, 24, 1473 (1965).
90. Brimacombe, R., Trupin, J., Nirenberg, M., Leder, P., Bernfield, M. and Jaocini, T., *Proc. Nat. Acad. Sci. U.S.*, 54, 954 (1965).
91. Tomkins, G.M., Gelehrter, T.D., Granner, D., Martin, D., Jr., Samuels, H.H. and Thompson, E.B., *Science*, 166, 1474 (1969).
92. Spirin, A.S., in A.A. Moscona and A. Monroy (Editors), *Current Topics in Developmental Biology*, Academic Press, New York, 1966, Vol. 1, P. 1.

93. Spirin, A.S., Eur. J. Biochem., 10, 20 (1969).
94. Cross, M. and Goldwasser, E., J. Biol. Chem., 246, 2480 (1971).
95. Henshaw, E.C., J. Mol. Biol., 36, 401 (1968).
96. Cartouzou, G., Poiree, J.C. and Lissitzky, S., Eur. J. Biochem., 8, 357 (1970).
97. Scherrer, K., Spohr, G., Granboulan, N., Morel, C., Grosclaude, J. and Chezzi, C., Cold Spring Harbor Symposia on Quantitative Biology, 35, 539 (1970).
98. Spohr, G., Granboulan, N., Morel, C. and Scherrer, K., Eur. J. Biochem., 17, 296 (1970).
99. Littau, V.C., Burdick, C.S., Alfrey, V.G. and Mersky, A.E., Proc. Nat. Acad. Sci. U.S., 54, 1204 (1965).
100. Littau, V.C., Alfrey, V.G., Fenster, J.H., Mersky, A.E., Proc. Nat. Acad. Sci. U.S., 52, 93 (1964).
101. Alfrey, V.G., Littau, V.C., Mersky, A.E., Proc. Nat. Acad. Sci. U.S., 49, 414 (1963).
102. Fenster, J.H., J. Cell Biol., 23, 32 (A) (1964).

FOR REFERENCE

NOT TO BE TAKEN FROM THE ROOM



CAT. NO. 23 012

PRINTED
IN
U.S.A.

COPY 1

