UPTAKE OF GLYCINE-1-^{14}C INTO THE ACTOMYOSIN AND COLLAGEN FRACTIONS OF DEVELOPING CHICK MUSCLE*

HEINZ HERRMANN, LEONARD LERMAN, AND BETTY N. WHITE

Laboratory of Chemical Embryology, Department of Pediatrics, University of Colorado Medical School, Denver, Colorado (U.S.A.)

Measurements of the accumulation of collagen[1] and of actomyosin[2] in the leg musculature of the developing chick showed that these proteins accumulate more rapidly than the bulk of the muscle proteins from the 12th day to the 18th day of development. Following this period the accumulation rates of both proteins decrease, with actomyosin maintaining a higher accumulation rate than collagen for several weeks during the post-hatching period[3,4]. As the next step in the analysis of protein formation in developing muscle, it became desirable to compare the rates of incorporation of tracer amino acids into collagen and actomyosin with the accumulation rates of these proteins. The results of a series of such measurements are given in this paper.

MATERIALS AND METHODS

Fertile eggs from a Hi-Line chick strain were purchased from a commercial hatchery and incubated at the laboratory at 37.5° C. Glycine-1-^{14}C was injected into the choriollantoic vein of the embryo following the procedures used previously at this laboratory[4]. In the hatched chick, the glycine solution was injected into the wing vein. The amount of glycine injected was 40 μg of glycine, corresponding to $4.4 \cdot 10^6$ c.p.m., dissolved in 0.02 ml of 0.7% saline and in the hatched chick, a 5 times larger amount (200 μg, $22 \cdot 10^6$ c.p.m.) dissolved in 0.1 ml. The 14-day embryo has a bloodvolume of about 2.4 ml[5]. From our determinations a blood glycine level of 60–70 μg/ml was found corresponding to a total glycine amount of about 143–168 μg. Therefore, by injection of the labelled glycine, the glycine level in the blood was raised by 25–30%. In the hatched chick a bloodvolume of 6 ml and a glycine level of 60 μg/ml give a 40% increase of the glycine level after injection of the labelled amino acid. The blood samples were removed from the allantoic vein with a bent sharpened glasspipette. The blood from tne hatched chick was collected after decapitation in a small oxalated glassbeaker.

The entire leg musculature of the embryos and samples of the main muscles of the leg of the chick were quickly dissected and minced, and aliquots of 100 and 200 mg weighed out on a torsion balance. The blood-samples and the smaller muscle aliquot were precipitated in 80% alcohol, the latter after homogenization with alcohol in a glass homogenizer for preparation of free muscle amino acids. The larger muscle samples were used for preparation of the protein fractions. Actomyosin plus myosin (actomyosin fraction) was prepared by extracting the muscle with a solution of 0.3 *M* KCl and 0.15 *M* phosphate of pH 6.5–6.7 and precipitation by dilution with 8 volumes of glass distilled water. The precipitate was centrifuged off, redissolved in the KCl–phosphate solution and reprecipitated for two more times. The final precipitate was extracted with cold and hot trichloracetic acid to remove nucleotides and coprecipitated nucleic acids and lipoids were removed with hot ether-alcohol. This was followed by an extraction with H_2SO_4–$HgCl_2$[6] to remove basic proteins, possibly coprecipitated as nucleoproteins. Finally, the precipitate was washed several times with a 1% glycine solution followed by six washings with distilled water. The protein residue

* Aided by grants from the National Institute of Blindness and Neurological Diseases (Grant No. B-549) if the National Institutes of Health, United States Public Health Service, and the Jane Coffin Childs Memorial Fund for Medical Research.

References p. 164.

remaining after removal of the actomyosin was extracted with 0.1 *N* NaOH and the collagen was obtained from the residue as described before[2]. By using this rigorous extraction procedure, it was attempted to remove possibly loosely adhering small molecular protein precursors (glutathione). Also, in a more recent series of experiments with explanted chick embryos, we found that only negligible traces of activity are liberated after treatment with ninhydrin from our unhydrolyzed proteins following this extraction procedure. The actomyosin and the collagen were hydrolyzed in 1 ml of 6 *N* HCl in sealed tubes at 140° for 4 h and the HCl was evaporated *in vacuo*.

The hydrolysate of the protein fractions and the evaporated alcohol extracts of blood and muscle were taken up in a bicarbonate solution and reacted with dinitro fluorobenzene[7] and the glycine derivative was separated chromatographically following the procedure of KROL[8]. An aliquot of the DNB glycine was used for the determination of the total glycine in the respective sample. The other aliquot was counted either in an inner gas counting tube (Woods Counting Laboratory) or with a gas flow counter with ultra-thin window (Nuclear, D 47). The values for total and tracer glycine in the blood were used only for calculation of a small correction (below 10%) converting the free muscle glycine into actual cell space glycine by subtraction of extracellular space glycine[4] assumed to be in equilibrium with the blood. For this purpose the differences found for tracer glycine in whole blood and in plasma were of no significance.

RESULTS

Measurements of the incorporation of glycine-1-^{14}C into the actomyosin and collagen fractions of the leg musculature of the developing chick gave the specific activities (S.A.) recorded in Table I. In the actomyosin fraction of the 14-day embryo the S.A. increases rapidly during the first half hour after injection of the tracer amino acid. After this initial period, the S.A. increases at a diminishing rate until it reaches a value of 37.4 two hours after the injection of the tracer. There is some fluctuation in the S.A. level during the 2–4 hour period and a definite continuous decrease follows this interval reaching a S.A. level of 23.7 after 24 hours.

The incorporation of glycine into collagen follows a strikingly different course. The uptake during the first half hour is very small. It increases rapidly from one to four hours after injection reaching a distinct maximum after about six hours which is followed by a slow decrease in activity comparable to the decrease of S.A. in the actomyosin fraction.

TABLE I

SPECIFIC ACTIVITIES OF GLYCINE (MEAN μg GLYCINE-1-^{14}C/μg GLYCINE-1-^{12}C · 10^3 ± S.E.) FOR ACTOMYOSIN, COLLAGEN, CELL SPACE, AND BLOOD OBTAINED AT DIFFERENT TIMES AFTER INJECTION OF TRACER GLYCINE

Time after injection (hours)	*Number of determinations*	*Actomyosin*	*Collagen*	*Cell space*	*Blood*
Embryos					
0.5	7	26.9 ± 1.7	0.591 ± 0.13	434.3 ± 36.6	1858.6 ± 281.2
1	16	30.5 ± 1.4	2.33 ± 0.1	549.6 ± 14.8	755.6 ± 41.3
2	14	37.4 ± 2.9	19.5 ± 1.2	296.4 ± 22.3	233.1 ± 12.6
4	6	34.5 ± 7.5	51.9 ± 5.0	105.7 ± 9.9	65.3 ± 3.5
6	6	31.5 ± 4.2	60.3 ± 4.9	50.6 ± 2.6	35.1 ± 2.4
8	8	40.1 ± 2.7	53.4 ± 3.2	21.8 ± 1.5	16.8 ± 1.2
12	8	29.8 ± 1.5	43.2 ± 2.1	12.3 ± 0.7	10.1 ± 1.2
24	8	23.7 ± 1.6	38.5 ± 2.3	6.5 ± 0.6	6.8 ± 1.8
Hatched chick					
0.5	15	3.97 ± 0.6	0.4 ± 0.03	761.5 ± 5.5	
1	7	9.4 ± 0.9	0.5 ± 0.074	406.6 ± 47.4	1281.2 ± 122.4
4	9	10.9 ± 2.1	3.1 ± 0.17	206.8 ± 19.3	94.1 ± 4.8

References p. 164.

The incorporation into the fractions of the hatched chick was measured so far only during a four hour period. From the data in Table I, it can be seen that during this time interval the increase in S.A. follows essentially the same course as in the embryo but at a much slower rate. The maximum level reached by the actomyosin fraction after one hour is about 1/4 of the figure found for the embryo, and in case of collagen of the hatched chick muscle the lag period does not end until sometime between 1 and 4 hours after injection compared to a lag period of ½ hour in case of the embryo.

For an interpretation of the protein S.A., the tracer content of the non-protein glycine (NPG) was determined. In the embryo, the NPG values were found to reach maximal levels during the 30–60 min period after injection. The actual maximum of NPG activity may have occurred between these first two timepoints. With the injected dose of tracer glycine only approximately adjusted proportionally to the body weight, higher NPG values were obtained in the hatched chick at the four hour interval than in the embryo while the one hour level was found to be higher in the embryo.

In comparing the NPG and protein activities one can see from the data in Table I that in the embryo, the initially higher NPG values decline to the collagen values about 6 hours and to the actomyosin value about 8 hours after injection. While the values for NPG activities and collagen activities thus intersect close to the peak of collagen activity, in case of actomyosin the S.A. of the protein levels off 7 hours before the NPG activity has fallen to the actomyosin level. In the hatched chick the period of measurement has not been sufficiently extended to establish the time of bisection of NPG and protein values.

DISCUSSION

The lag period in the collagen activity curves of the embryo and the hatched chick indicates[9] that the collagen isolated by our procedure is not formed directly from NPG but from some intermediary precursor, possibly a procollagen as described by HARKNESS *et al.*[10], which is removed in the course of our collagen preparation. Therefore, it is probably coincidental that the observed figures for collagen activity reach the NPG level near the maximum value of the former and is not indicative of a direct precursor-product relationship of NPG and collagen. On the other hand, the rapid initial increase in the specific activity of the total actomyosin fraction and its leveling off before reaching the NPG values indicates that in case of actomyosin, a small but rapidly incorporating portion of this fraction was not removed by our preparative procedure. An "active" myosin has already been postulated by SHEMIN AND RITTENBERG[11]. In our case, the actomyosin as it is formed, (possibly on the microsomal surfaces) would represent a small actomyosin fraction which equilibrates rapidly with free cell glycine comparable to the procollagen fraction. This newly formed actomyosin is then deposited in the structures of the muscle filaments corresponding to the extracellular deposition of collagen. In this state it equilibrates only at a negligible rate with the free cell glycine. The assumption of a small but actively incorporating and of a larger inert portion in our actomyosin would explain why the specific activity of this fraction does not continue to increase until it reaches the values of the NPG.

For the same reason, the initial increase in actomyosin activity is apparently much faster than the collagen activity, in spite of the fact that the accumulation of collagen on the 14th day is somewhat faster than the accumulation of the actomyosin

References p. 164.

fraction prepared in the present measurements. On the other hand, the similar rates in the decline of the collagen and actomyosin activities due to formation of minimally labelled proteins suggest that this decline is a better parameter for the comparison of accumulation rates and activity changes.

Alternative explanations would seem to fit the observed data less well. For example, a difference in the incorporation rates of myosin components[12] would still require an increase in the total actomyosin activity until it reaches the NPG value. In this connection, it should be mentioned that preliminary fractionations of our crude actomyosin fraction into actin and myosin do not indicate that either one of these two fractions, by itself, is responsible for this rapid uptake.

The specific activities for actomyosin and collagen given in our Table I represent obviously the ratio of the amounts of tracer glycine added per time unit over the protein glycine present per freshweight unit of mucle tissue at the time of the experiment. The fact that the specific activities increase much slower in the proteins of the hatched chick as compared to the embryo can mean that uptake of tracer is slower, that the amount of protein per 100 mg muscle freshweight is higher, or both. As pointed out above, the amount of actomyosin and collagen increases rapidly from the 14th to the 28th day of development, and therefore at least a large portion of the decrease in specific activities can be accounted for by the increase in protein per muscle freshweight.

The measurements reported in this paper have been discontinued for the time being until a more complete fractionation of embryonic actomyosin can be carried out. Anomalies in the fractionation of the embryonic actomyosin observed during the attempts to separate actomyosin precursors will be reported later.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. DANIEL STEINBERG, National Institute of Health for a critical discussion of this manuscript.

Thanks are due to Mrs. M. COOPER for competent assistance in carrying out part of the determinations.

SUMMARY

The level of glycine-1^{14}C was determined in the blood and in the nonprotein glycine, in the collagen fraction and the actomyosin fraction of muscle tissue of 14-day chick embryos and of freshly hatched chicks.

The initial rate of incorporation of the tracer into the collagen fraction shows a lag period and remains significantly lower than the incorporation rate into the actomyosin in spite of the similarity in the accumulation rates of the two protein fractions. Possible interpretations of these differences are discussed.

REFERENCES

1 A. CZAPO AND H. HERRMANN, *Am. J. Physiol.*, 165 (1951) 701.
2 H. HERRMANN AND S. R. BARRY, *Biochim. Biophys. Acta.*, 55 (1955) 526.
3 I. R. KONIGSBERG AND H. HERRMANN, *Biochim. Biophys. Acta.*, 55 (1955) 534.
4 H. HERRMANN, B. N. WHITE AND M. COOPER, *J. Cellular Comp. Physiol.*, 49 (1957) 227.
5 Y. YOSPHA-PURER, J. FENDRICH AND A. M. DAVIES, *Am. J. Physiol.*, 175 (1953) 178.
6 A. E. MIRSKY AND A. W. POLLISTER, *J. Gen. Physiol.*, 30 (1946) 117.
7 F. SANGER, *Biochem. J.*, 39 (1945) 507.
8 S. KROL, *Biochem. J.*, 52 (1952) 227.
9 S. ARONOFF, *Techniques of Radiobiochemistry*, Ames State College Press, 1956.
10 R. D. HARKNESS, A. M. MARKO, H. M. MUIR AND A. NEUBERGER, *Biochem. J.*, 56 (1954) 558.
11 D. SHEMIN AND D. RITTENBERG, *J. Biol. Chem.*, 157 (1944), 401–421.
12 S. F. VELICK, *Biochim. Biophys. Acta.*, 20 (1956) 228.

Received July 23rd, 1957