The *in Vitro* Biosynthesis of Hemoglobin A_{Ic}*

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ABSTRACT: Hemoglobin A_{Ic} is synthesized *in vitro* concurrently with hemoglobin A, which is the major component in normal adult humans. A_{Ic} is thus a normal constituent of all red cells, irrespective of their physiological age. By studying the incorporation of radioactive L-[^{14}C]valine into these hemoglobins, it is demonstrated that A_{Ic} and A_{II} are either (1) not interconvertible, (2) in rapid equilibrium, or (3) though interconvertible at moderate rates, any formation of A_{Ic} from A_{II} (or *vice versa*) is just offset by the reverse process. Experiments are suggested to distinguish among these three possibilities. One possible function for hemoglobin A_{Ic} in the red cell is considered in light of its presently known structure. A theoretical treatment of protein biosynthesis has been developed which includes a method for correcting the experimental data for nonconstant ribosomal activity and for the interconversion of one protein into another during the biosynthesis. This theory is used to study the biosynthesis of hemoglobins A_{Ic} and A_{II}.

Hemoglobin A_{Ic} is the Schiff base between one molecule of hemoglobin A_{II} and one molecule of an aldehyde or ketone R=O. The latter is condensed with the N terminus of one of the β chains; its structure has been discussed in the preceding paper (Holmquist and Schroeder, 1966). It is the purpose of this paper to present some aspects of the biosynthesis of this interesting hemoglobin and of its possible function in the red cell.

Several questions of biological interest can be raised about hemoglobin A_{Ic}. What is its origin in the red cell? Is it synthesized by the cell independently of the major hemoglobin A_{II}? Is it a degradation or metabolic product of A_{II} or *vice versa?* Conceivably, each of these processes may be occurring; if so, what are their rates? Finally, what is the relationship between the amount of A_{Ic} in an erythrocyte and the physiological age of that erythrocyte? Is A_{Ic} only to be found in older red cells, or might it also be a normal constituent of younger red cells? An attempt has been made to answer some of these questions by studying the *in vitro* incorporation of radioactive L-[^{14}C]valine into hemoglobins A_{Ic} and A_{II}.

Experimental Section

Source of Reticulocyte-Rich Red Blood Cells. The blood from two donors was used in these experiments. Donor A was a 27-yr-old Negro woman who had a brisk hemolytic anemia that had arisen from mechanical damage to the red cells after insertion of an artificial heart valve of the Starr–Edwards type for treatment of an incompetent aortic valve. The patient's hematocrit and reticulocyte count were each 25%. Her blood was drawn into a heparinized syringe at 25°, transferred to a chilled test tube, and transported at 0° to the laboratory. Donor B was a 70-yr-old Caucasian woman with idiopathic cold agglutinin disease. The hematocrit and reticulocyte count of this patient were 19 and 25%, respectively. Her blood was drawn into a heparinized syringe that had been prewarmed to 37°. The blood was immediately centrifuged at 37°, the serum was removed and discarded, and the cells were washed twice at 37° with NKM[1] solution (NaCl, 9 g/l.; KCl, 0.37 g/l.; $MgCl_2 \cdot 6H_2O$, 1.02 g/l.). The washed cells were transported to the laboratory at 0°.

Incubation and Chase Experiments. The procedures which were used in preparing the donors' cells for incubation and in the incubation itself were identical with those that Philipps (1965) found to be suitable for *in vitro* incubation of rabbit reticulocytes with [^{14}C]amino acids with the following exceptions. Because of the small amount of blood that was available, the scale of the experiments was reduced by a factor of 4. Unfiltered and filtered normal human serum (Hyland Laboratories, Los Angeles), rather than anemic plasma, were used with reticulocytes from donors A and B, respectively, and the vitamin solution was omitted from the latter. The reagent mixture contained 10,000 units of penicillin G and 50 mg of streptomycin sulfate/500 ml rather than 400 units and 2 mg, respectively. Because radioactive valine rather than radioactive leucine was used, the amino acid mixture contained 6.3 mmoles/l. of L-leucine instead of 8 mmoles/l. of L-valine. Finally, the incubation was started by adding 12.75 μmoles of uniformly labeled L-[^{14}C]valine (rather than L-[^{14}C]-

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[1] Abbreviations used: NKM, NaCl–KCl–$MgCl_2$; STM, sucrose–KCl–$MgCl_2$ in Tris.

TABLE I: Specific Activities[a] of Hemoglobins Biosynthesized *in Vitro*.

Donor A				Donor B			
Time of Incubn (min)	A_{Ic}	A_{II}	A_{Ic}/A_{II}[c]	Time of Incubn (min)	A_{Ic}	A_{II}	A_{Ic}/A_{II}[d]
	44.8[b]	70.5			23.3	22.5	
15.5	44.7	59.0		45	18.0	13.3	
	70.7	63.7			20.0	10.9	
	Av 53.4 ± 10.1	64.4 ± 3.9	0.83 ± 0.16		Av 20.4 ± 1.8	15.6 ± 4.1	1.32 ± 0.37
	330	352			21.9	25.6	
30	204	385		90	21.0	22.7	
	284	420			17.4	23.4	
	Av 273 ± 43	386 ± 23	0.71 ± 0.12		Av 20.1 ± 2.3	23.9 ± 1.0	0.84 ± 0.10
	208	515		180	59.7	42.8	
60	184	504			45.4	52.4	
	273	550			Av 52.5 ± 6.8	47.6 ± 1.1	1.10 ± 0.18
	Av 222 ± 32	523 ± 16	0.42 ± 0.06				
Chase of 15-min Incubn				Chase of 90-min Incubn			
Duration of chase				Duration of chase			
	31.9	68.4			22.1	26.9	
15	39.9	68.3		45	19.7	18.1	
	42.3	76.0			22.8	23.6	
	Av 38.1 ± 3.7	70.9 ± 3.0	0.54 ± 0.06		Av 21.5 ± 1.1	22.9 ± 3.0	0.94 ± 0.13
	68.7	96.6					
30	102.5	117.6					
	182.2	122.5					
	Av 117.8 ± 39.3	112.2 ± 9.3	1.05 ± 0.36				

[a] Counts per minute per milligram of Hb; not corrected for counter efficiency. [b] These three values throughout this table, from top to bottom, are the specific activities of samples from the front, middle, and tail of the chromatographic peak. [c] Average value of A_{Ic}/A_{II} for all incubations and chases of donor A, 0.71 ± 0.17. [d] Average value of A_{Ic}/A_{II} for all incubations and chases of donor B, 1.05 ± 0.14.

leucine) with a specific activity of 6.5 mc/mmole. The temperature of the incubation was 37°.

In studying the kinetics of incorporation, incubations for different times were carried out in separate flasks rather than by taking aliquots from a single flask at different times; for example, for donor A in Table I, the 15-min incubation was done in one flask, the 30-min incubation in a second, and the 60-min incubation in a third. In view of the small amount of blood that was available, this procedure had the advantage that if an incubation in any one flask failed, the other flasks would remain unaffected.[2] The time of incubation was varied from 15 min to 3 hr. For periods longer than 3 hr the biological integrity of the *in vitro* system is uncertain, whereas Philipps (1965) has given evidence that for periods up to 2 hr the biological integrity of the reticulocyte is unimpaired, at least with respect to hemoglobin synthesis. The lower limit (15 min) was set by theoretical considerations (see below).

The chase was conducted by removing a 5.0-ml aliquot of the red cell suspension from the incubating mixture and adding the aliquot to an equal volume of the complete reaction mixture (without any L-[^{14}C]-valine) which was 0.4 F in L-[^{12}C]valine. The ratio of nonradioactive to radioactive valine was 700–800. After the incubation or chase had proceeded for the desired length of time, the reaction was stopped by adding one-half volume of NKM at 0° to the flask and immediately placing it in ice water.

Isolation of Radioactive Ribosomes and Hemoglobin. The lysate, which contained the radioactive ribosomes and hemoglobin, was obtained from the incubation or chase mixture as described by Philipps (1965). After the ribosomes had been removed from the lysate by centrifugation through STM solution (0.5 F sucrose, 0.04 F KCl, 0.0015 F MgCl in pH 7.0 Tris–HCl that was 0.01 F in Tris) (Philipps, 1965), the supernatant, which contained the hemoglobin, was saturated with carbon monoxide. The solution, after dialysis at 2° *vs.* three changes of developer 4 (Allen *et al.*, 1958), was

[2] Six out of eight incubations were successful. The occasional failure reflects the variable quality of *in vitro* biological systems.

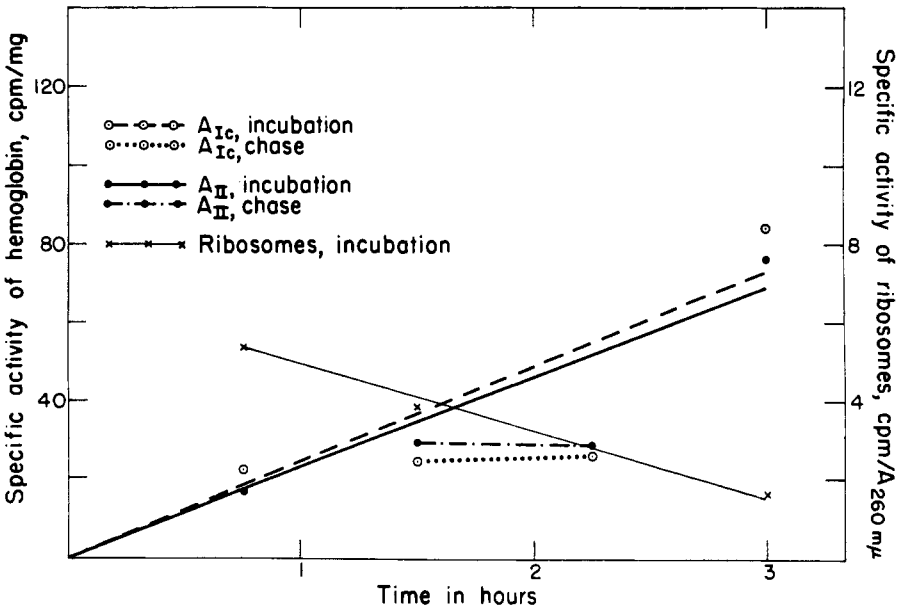

FIGURE 1: The experimentally observed incorporation of L-[^{14}C]valine into hemoglobins A_{Ic} and A_{II}. See text for discussion.

concentrated at 2° in collodion bags,[3] and its absorbance was determined at 260, 280, 415, and 520.5 mμ. The last wavelength was used to calculate the concentration of hemoglobin (Holmquist and Vinograd, 1963). In order to obtain purified fractions of hemoglobins A_{Ic} and A_{II}, 1 ml of this solution, containing 40–100 mg of hemoglobin, was chromatographed at 6.8° on a 1 × 30 cm IRC-50 cation exchange column with developer 4 (Allen *et al.*, 1958; Clegg and Schroeder, 1959; Jones, 1961).

Measurement of the Specific Activity of the Ribosomes and Hemoglobin. The specific activity of the ribosomes was measured as described by Philipps (1965), except that sucrose gradient analyses of the ribosomes were not made. The specific activities of purified hemoglobin components were determined as for the ribosomes except that the precipitation of the protein with 10% trichloroacetic acid was carried out at 90° for 1 hr. This precaution ensured the hydrolysis of any radioactive aminoacyl-RNA which might have been present; such hydrolyzed ribonucleic acid (RNA) would pass through the Millipore filter on which the precipitated hemoglobin was collected. The precaution probably was not necessary because of the prior purification of the hemoglobin on IRC-50, but it was done as an added safeguard against contaminating the hemoglobin samples with nonhemoglobin radioactive components. No corrections were made for counter efficiency.

[3] Membrane filter; type, collodion bags CB; porosity, <5 mμ; size, 8 ml; Membranfiltergesellschaft, Göttingen, Germany.

Results

Ribosomal Specific Activity. Ribosomal pellets that were isolated as described were red. This indicated contamination with hemoglobin (and hence possibly with other proteins). Rabbit ribosomes which have been isolated as described are largely free from contaminating hemoglobin (Philipps, 1965), although recently G. Philipps (private communication) has also observed a contamination of rabbit ribosomes with hemoglobin. Different ribosomal pellets differed greatly in the extent of contamination with hemoglobin. Between 2 and 95% of the absorbance at 260 mμ was due to hemoglobin. This variability was observed even in pellets from the same sample of blood and was not obviously related to the duration of the incubation. The contamination could mean that heme was added to the polypeptide chain while it was still on the ribosome (Granick and Levere, 1964; Winterhalter, 1964), or that the conditions were not optimal for isolating human ribosomes from reticulocytes. For the total of 12 samples that were examined, the ratio A_{280}/A_{260} (after correction for contaminating hemoglobin) was 0.80 ± 0.08 for human ribosomes. In view of the large variability in the amount of contaminating hemoglobin, the constancy of this ratio is remarkable and suggests that contamination by other proteins is small.

The ribosomal specific activities (corrected for contaminating hemoglobin) from one set of incubations are plotted as a function of time in Figure 1. The decrease of ribosomal activity with time was observed for both donors. Such a decrease has been observed by others (Allen and Schweet, 1962; Goodman and Rich, 1963; Hardesty *et al.*, 1963; Wettstein *et al.*, 1963). Experiments by Philipps (1965) suggest that the de-

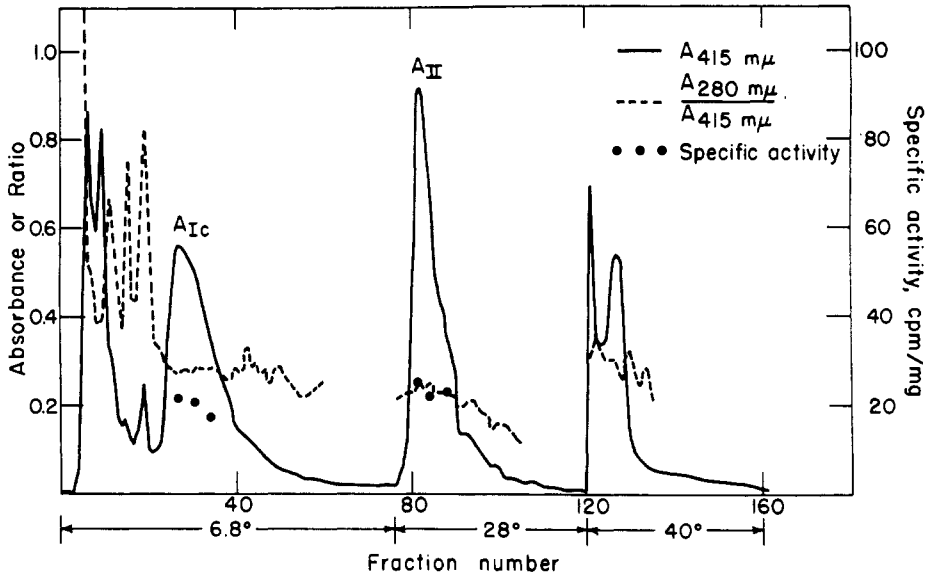

FIGURE 2: The isolation and specific activities of hemoglobins A_{Ic} and A_{II} from the 90-min incubation of the reticulocytes from donor B (Table I). Column size 1 × 30 cm. The fraction size was 2 ml for fractions 1–20, and 4 ml for fractions 21–160. Flow rate 6 ml/hr. The absorbance of the A_{II} peak (only) has been reduced by a factor of 21.7. See text for discussion.

crease may be due to nonoptimal conditions during the incubation.

Specific Activities of Hemoglobins A_{Ic} and A_{II}. The isolation of radioactive A_{Ic} and A_{II} is shown in the chromatogram of Figure 2. The purity of each of these hemoglobins is attested to both by the constancy of the ratio of absorbances at 280 and 415 mμ and by the constancy of the specific activities across a chromatographic zone.

The specific activities of the purified hemoglobins from the two sets of incubations are given in Table I. The degree of incorporation of radioactive L-valine was appreciably different for the two donors. The lower incorporation by the reticulocytes of donor B may be due to the fact that filtered, rather than unfiltered, serum was used, and to the fact that the vitamins were omitted. The need to wash the cells of donor B at 37° to remove serum agglutinins prior to the incubation may also have reduced the ability of the cells to synthesize hemoglobin. Three facts should be noted in this table: first, both hemoglobins A_{Ic} and A_{II} are radioactive; second, the ratio of the specific activities of these two hemoglobins is approximately unity; and third, during the chase experiments there is no appreciable change in the specific activity of either A_{Ic} or A_{II}.[4]

Conclusions

The fact that both A_{Ic} and A_{II} become radioactive during the incubation shows that A_{Ic} is *not* a product from the degradation of hemoglobin A_{II} and is therefore not a component of older cells only. Rather, A_{Ic} is a normal biosynthetic product of the reticulocyte.

The fact that the synthesis of A_{Ic} is concurrent with that of A_{II} implies that either the ketone or aldehyde R=O is attached to the β chain while the latter is still on the ribosome, or that R=O is attached to the β chain relatively soon after its release therefrom.

Because the specific activities of A_{Ic} and A_{II} are equal within the experimental error at any given time,[5] and because no significant change in the specific activities of A_{Ic} or A_{II} occurs during the chase experiments, one of the following three possibilities is implied: (1) A_{Ic} and A_{II} are not rapidly interconvertible, (2) A_{Ic} and A_{II} are in rapid equilibrium, or (3) though interconvertible at moderate rates, any formation of A_{Ic} from A_{II} (or *vice versa*) is just offset (by "numerical accident") by the reverse process.

Theoretical

In this section a theoretical framework is developed through which the above data can be more quantitatively examined. The theoretical treatment is cer-

[4] The 30-min chase of the 15-min incubation of the reticulocytes from donor A appears to contradict this statement. However, the observed high values of the specific activities of A_{Ic} and A_{II} cannot be correct because the average specific activity of $A_{Ic} + A_{II}$ cannot increase appreciably during a chase experiment. The cause is probably an unexplained decrease in the absorbance of these samples. The ratio A_{Ic}/A_{II} (1.05) would be unaffected by such a decrease in absorbance.

[5] This statement is true for seven of the nine incubations in Table I. The two exceptions are the 60-min incubation and the 15-min chase. In view of the sensitivity of this system (see footnote 2), we do not believe these two exceptions invalidate the general conclusion.

tainly more thorough than the data are accurate. However, the concepts which are developed are generally applicable to other biological systems and may be useful for studying the latter. In order to establish a factual foundation for some of the assumptions on which this framework is based, a very brief summary of current knowledge about those aspects of protein synthesis that are relevant for our purposes is given in the following paragraph. More detailed accounts and references to earlier work can be found in Watson (1964), Glowacki and Millette (1965), and Philipps (1965).

In vitro protein synthesis in reticulocytes, liver, and HeLa cells has been shown to take place with the aid of intracellular particles called polysomes. It is probable that in the healthy reticulocyte the pentasome is the major site of protein synthesis. The rate at which this synthesis proceeds is linearly proportional to the number of and the synthetic quality of these polysomes. This rate decreases with cell age or mishandling.

Incubation Experiments. THEORY. If A_{Ic} and A_{II} are made independently at constant rates A and B, respectively, and if each is made from the other at a rate proportional to the amount of the other present,[6] then

$$\frac{dA_{Ic}}{dt} = A + kA_{II} - k_-A_{Ic}$$
$$\frac{dA_{II}}{dt} = B + k_-A_{Ic} - kA_{II} \quad (1)$$

where k is the proportionality constant for the formation of A_{Ic} from A_{II}, and k_- that for the formation of A_{II} from A_{Ic}. If at time zero no A_{Ic} or A_{II} is present, the solution to these equations is

$$A_{Ic} = \frac{(Ak_- - Bk)}{(k + k_-)^2}[1 - e^{-(k+k_-)t}] + \frac{(A + B)}{k + k_-}kt$$
$$A_{II} = \frac{(Bk - Ak_-)}{(k + k_-)^2}[1 - e^{-(k+k_-)t}] + \frac{(A + B)}{k + k_-}k_-t \quad (2)$$

These equations would apply, for example, to the *in vitro* synthesis of radioactive A_{Ic} and A_{II} if at time $t = 0$ radioactive L-valine were added to a suspension of reticulocytes in an appropriate nutritive medium. The equations allow one to calculate the amounts of radioactive A_{Ic} and A_{II} present at time t provided that the assumptions on which the equations are derived hold for the *in vitro* system.

Normally, an incubation experiment in principle is capable of completely determining the magnitudes of A, B, k, and k_-: A and B, respectively, are given by the initial slope of plots of the amounts of radioactive A_{Ic} and A_{II} formed as a function of time, and the rate constants k and k_- can then be calculated from the deviation of the plots from linearity. However, in four cases, only partial information about these constants can be obtained. Two of these occur if in the power series expansions of eq 2 the coefficients of all terms nonlinear in t vanish. This can happen in two ways

$$k/k_- = A/B \quad (3a)$$

$$k = k_- = 0 \quad (3b)$$

In these cases, A and B are still uniquely determined as before but k and k_- are ill defined to the extent of the above two possibilities. The other two exceptions occur when k or k_- or both become very large. Then,

$$A_{Ic} = \frac{A + B}{(1 + k_-/k)}t$$
$$A_{II} = \frac{A + B}{(1 + k/k_-)}t \quad (4)$$

The case where both k and k_- become large corresponds to a rapid chemical equilibrium between A_{Ic} and A_{II}. The ratio k/k_- is the equilibrium constant and is equal to A_{Ic}/A_{II} at *any* time. From this, $(A + B)$, but not A and B individually, can be found. The case where either k or k_-, but not both, becomes large can be recognized by the fact that then the amount of radioactive A_{II} or A_{Ic}, respectively, which is present at any time will be zero. In this case the only information that can be extracted is the sum $(A + B)$. Finally, the case of rapid chemical equilibrium cannot be distinguished (by kinetic experiments) from the possibility of eq 3a; similarly, the case where either k or k_-, but not both, becomes large cannot be distinguished from the possibility of eq 3b where B or A, respectively, is zero.

Chase Experiments. THEORY. Although in principle an incubation experiment is capable of answering the kinetic questions that have been posed, in practice the accuracy of the data, its incompleteness, or the fact that the experiment has not been or cannot be continued for a long enough time may cause the deviations from linearity to be unobservable. If this is the case, one cannot extract the maximum of information by an analysis of the above type. Sometimes these difficulties can be reduced by conducting a chase experiment: if during the course of an incubation a large excess, say one-thousandfold, of nonradioactive L-valine is added to the incubating mixture, all but a negligible fraction of the hemoglobin which is synthesized after that time will be nonradioactive. Thus one can follow the changes in the amount of radioactive A_{Ic} and A_{II} that are already present under the assumption $A = B = 0$. Equation 1 applies as before. If the amounts of radioactive A_{Ic} and A_{II} at the beginning of the chase are A^1_{Ic} and A^1_{II}, respectively, then at any later time t, measured from the beginning of the chase, the amounts of each will be

[6] No experimental basis existed for the first-order kinetics which have been assumed for the interconversion of hemoglobins A_{Ic} and A_{II}. These kinetics were chosen for their simplicity and reasonableness in the light of the chemical structures of A_{Ic} and A_{II}.

$$A_{Ic} = \frac{A^1_{Ic} + A^1_{II}}{k + k_-} k + \frac{A^1_{Ic} k_- - A^1_{II} k}{k + k_-} e^{-(k+k_-)t}$$

$$A_{II} = \frac{A^1_{Ic} + A^1_{II}}{k + k_-} k_- - \frac{A^1_{Ic} k_- - A^1_{II} k}{k + k_-} e^{-(k+k_-)t} \quad (5)$$

The usefulness of these equations lies in the fact that in their power series expansion in t, the coefficient of t^{n-1} will give the same amount of information as the coefficient of t^n in an incubation experiment. The sensitivity of the experiment is thus increased by an order of magnitude.

Correction for Nonconstant Ribosomal Activity. In the derivation of the above equations, a constant rate of hemoglobin synthesis has been assumed. This assumption has been verified experimentally when the number of polysomes and their activity have remained constant (see earlier). A complication which is normally present to some degree in an actual incubation is the decrease in ribosomal activity over the period during which the experiment is conducted. This is of course reflected in a decreased rate of protein synthesis. If the ribosomal activity can be determined independently of the amount of protein synthesized, then the amount of protein which is actually made during a given time can be corrected to that which would have been made had the ribosomal activity remained constant. It is this corrected value that should be compared to the theoretical value that is calculated from eq 2.

Let the ribosomal activity **a** be given as a function of time by the equation

$$\mathbf{a} = a - \sum_1^n b_i t^i$$

The constants a and b_i can be determined by fitting the experimental points by the method of least squares or by some other method. The actual rates **A** and **B** at which the ribosomes are synthesizing hemoglobin A_{Ic} and A_{II}, respectively, are then

$$\mathbf{A} = c\mathbf{a} = ca - c\sum_1^n b_i t^i = A - c\sum_1^n b_i t^i$$

$$\mathbf{B} = d\mathbf{a} = da - d\sum_1^n b_i t^i = B - d\sum_1^n b_i t^i$$

where c and d are constants of proportionality, and $A = ca$ and $B = da$. It should be noted that A and B (but not **A** and **B**) have the same meaning as in the preceding sections. A and B are the constant rates at which A_{Ic} and A_{II}, respectively, would be synthesized by the polysomes if all the b_i were zero, *i.e.*, if the polysomal activity remained constant throughout the incubation.

Let A_{Ic} and A_{II} represent, as before, the amount of radioactive A_{Ic} and A_{II} which would be present at time t if the ribosomal activity remained constant during the incubation, and let A^0_{Ic} and A^0_{II} represent the amounts of radioactive A_{Ic} and A_{II} which are actually present at time t because the ribosomal activity decreases during the incubation. In the latter case the net rates at which A_{Ic} and A_{II} are being synthesized at time t is

$$\frac{dA^0_{Ic}}{dt} = \mathbf{A} + kA^0_{II} - k_-A^0_{Ic}$$

$$\frac{dA^0_{II}}{dt} = \mathbf{B} + k_-A^0_{Ic} - kA^0_{II} \quad (6)$$

where k and k_- have the same meaning as in eq 1. These equations reduce to eq 1 if all the b_i are zero. When the b_i are not all zero, the equations can be solved without approximation by the "Method of Undetermined Coefficients" (Rainville, 1949). We shall give the solution only for the case where the ribosomal activity decreases linearly[7] with time, *i.e.*, $b_1 \neq 0$, and $b_i = 0$ for $i > 1$ so that

$$\mathbf{a} = a - b_1 t$$

With this assumption, the solution to eq 6 is

$$A^0_{Ic} = A_{Ic} - \Delta_1 - k\Delta_2$$

$$A^0_{II} = A_{II} + \Delta_1 - k_-\Delta_2 \quad (7)$$

where[8]

$$\Delta_1 = \frac{b_1(Ak_- - Bk)}{a(k + k_-)^3}\{(k + k_-)t - [1 - e^{-(k+k_-)t}]\}$$

$$\Delta_2 = \frac{b_1(A + B)}{2a(k + k_-)} t^2$$

In practice, the simplest way to use the above equations is to determine A and B experimentally as described earlier[9] and to guess reasonable values of k and k_-. A^0_{Ic} and A^0_{II} are then calculated from eq 7. If the correct values of k and k_- have been chosen, the calculated values of A^0_{Ic} and A^0_{II} will agree to within the experimental error with the observed values if the basic assumptions are valid for the experimental system under investigation.

[7] From a calculational viewpoint it is simpler and quantitatively just as satisfactory to decompose a nonlinear decrease in ribosomal activity into several linear functions, each of which approximates the actual function for a particular period of time. Further, the accuracy with which the decrease in ribosomal activity can be measured sometimes does not justify anything more than a linear approximation.

[8] The use of the symbol "Δ" is not meant to imply that the corrections are small.

[9] At $t = 0$, Δ_1 and Δ_2 as well as their time derivatives are zero, so that A and B can still be obtained from the initial slope of a plot of the amount of radioactive A_{Ic} and A_{II} present as a function of time.

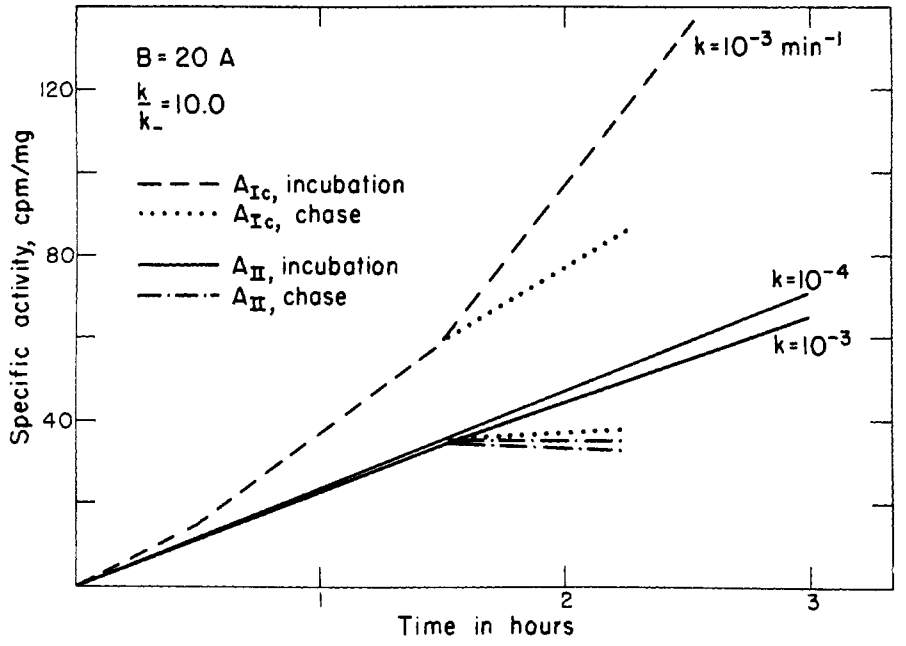

FIGURE 3: Predicted behavior for an incubation and chase experiment in which there is a *moderate* net synthesis of A_{Ic} from A_{II}. See text for discussion.

For the exceptional cases summarized in eq 3 and 4, eq 7 reduces to the simpler form

$$A_{Ic}^{0} = A_{Ic}\left(1 - \frac{b_1}{2a}t\right)$$
$$A_{II}^{0} = A_{II}\left(1 - \frac{b_1}{2a}t\right) \qquad (8)$$

The exceptional cases can be recognized in practice from the fact that either the specific activities[10] of A_{Ic} and A_{II} are equal for *all* times t or that the specific activity of either A_{Ic} or A_{II} is zero. In all these cases, the plot (after correction for changes in ribosomal activity) of the specific activity of A_{Ic} or A_{II} *vs.* time will be linear. This follows, with a little algebra, directly from eq 2.

Illustration of the Behavior Predicted by the Theory. Figures 3 and 4 show the predicted (eq 2) behavior for two hypothetical incubation and chase experiments. In both experiments the rate of synthesis of A_{II} by the ribosomes has been assumed to be 20 times the rate of synthesis of A_{Ic} because the cellular concentrations are in approximately this ratio (Clegg and Schroeder, 1959). In Figure 3 a moderate, and in Figure 4 a slight net synthesis of A_{Ic} from A_{II} has been depicted.[11] The specific activity units in these figures are arbitrary, though for comparison with the experimental results they have been assigned the numerical values shown. In both figures the important consideration is the shape of the curve for A_{Ic} with respect to the corresponding curve for A_{II}.

In Figure 3, an appreciable interconversion of A_{II} to A_{Ic} is assumed ($k/k_- \gg 1$). At $k = 10^{-4}$ min^{-1}, the curves for A_{Ic} and A_{II} are coincident but when $k = 10^{-3}$ min^{-1}, the divergence is very marked and increases rapidly as k becomes greater. The chase is represented as starting at 1.5 hr and continuing for 45 min. The chase curve for A_{Ic} at $k = 10^{-3}$ min^{-1} has a definitive positive slope because of the interconversion of A_{II} to A_{Ic}. On the other hand, the chase curve for A_{II} remains essentially horizontal because of the relative proportions of A_{II}/A_{Ic}. It should be noted that even if $k/k_- \ll 1$, there can still be a net synthesis of A_{Ic} because of the relatively large amount of A_{II} that is always present (Figure 4). However, in this instance, the divergence between the curves for A_{Ic} and A_{II} is less, and there is little change in specific activity of A_{Ic} during the chase.

Figures 3 and 4 also show that if there is only a moderate interconversion of A_{Ic} and A_{II}, an incubation of <1 hr is not likely to detect the interconversion because at shorter times the curves are not sufficiently divergent. It is for this reason that the incubations were conducted for periods varying from 15 min to 3 hr.

Correlation between Theory and Experiment

Because experimentally the specific activities of A_{Ic} and A_{II} are equal for all times t, the data can be described by one of the exceptional cases that have been discussed in the section on theory. Thus, the specific activities of A_{Ic} and A_{II}, after correction for the de-

[10] All equations have been derived in terms of *amounts* (grams, moles, etc.).

[11] If there is a net synthesis of A_{II} from A_{Ic} the curves for A_{Ic} then fall *below* those for A_{II}.

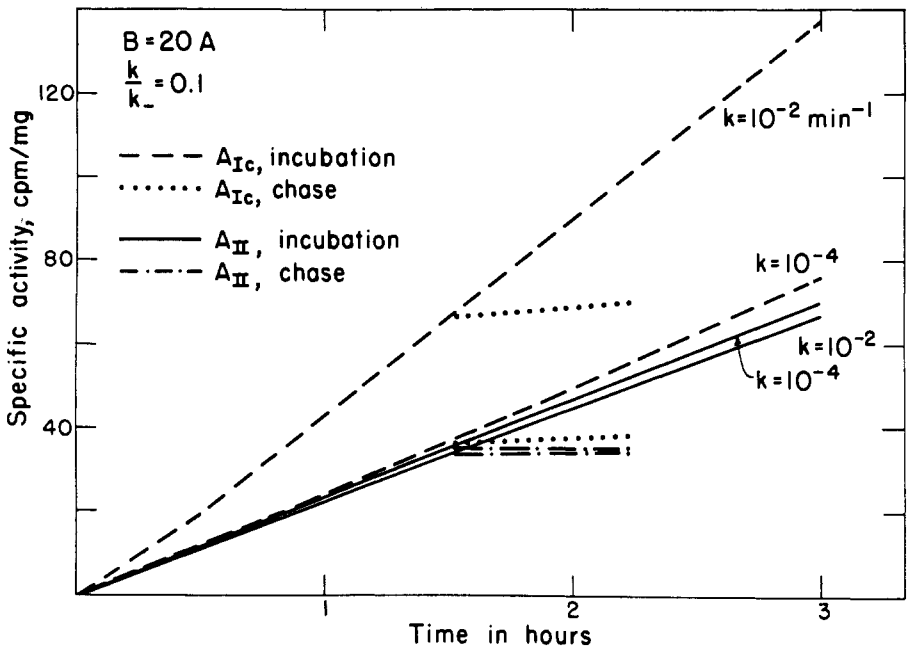

FIGURE 4: Predicted behavior for an incubation and chase experiment in which there is a *slight* net synthesis of A_{Ic} from A_{II}. See text for discussion.

crease in ribosomal activity, would be expected to increase linearly with time.

The primary data of donor B in Table I are plotted in Figure 1. The plotted points have been corrected for the decrease in ribosomal activity (eq 8) during the incubation, and a straight line, constrained to go through the origin, has been fitted to these points by the method of least squares. The predicted linearity is at least approximately borne out by the experimental data. The deviations of some experimental points from the least-squares line are larger than can be explained by the errors given in Table I. This may result from the fact that the incubations for different times were done in separate flasks (see Experimental Section). Lesser deviations might result if aliquots were taken from a single flask.

If the equality of specific activities of A_{Ic} and A_{II} is time independent, eq 2 implies one of the following: A_{Ic} and A_{II} are not rapidly interconvertible (eq 3b), are in rapid equilibrium (eq 4), or are interconvertible at moderate rates, which are such that no net synthesis of either results from the interconversions (eq 3a). These are precisely the three conclusions that were reached in an earlier section from experimental considerations alone. Noninterconvertibility would imply that the rates at which A_{Ic} and A_{II} are synthesized by the ribosomes are proportional to their average cellular concentrations. Examples are known where the rate of hemoglobin synthesis both is (Heywood *et al.*, 1964) and is not (Burka and Marks, 1964) proportional to the cellular concentration. A rapid equilibrium between A_{Ic} and A_{II} would imply only that the total rate (*i.e.*, $A_{Ic} + A_{II}$) of hemoglobin synthesis is proportional to the total ($A_{Ic} + A_{II}$) average cellular concentration. *A priori* there is no reason to throw out the numerical coincidence which is described by eq 3a; there may exist in the cell a good reason for such a coincidence.

With respect to the two possibilities that A_{Ic} and A_{II} may be either noninterconvertible or in rapid equilibrium, the "dynamic equilibrium" hypothesis of Guidotti *et al.* (1963) together with the fact that A_{Ic} and A_{II} *are* isolable by column chromatography would favor the former. However, it should be recognized that in the cell, conditions might favor an equilibrium: there might exist an enzyme "A_{Ic}–A_{II} transferase," for example, analogous to the acetokinase–hemoglobin F_I–hemoglobin F_{II} system investigated by Marchis-Mouren and Lipmann (1965). If the true situation lies somewhere in between noninterconvertibility and rapid equilibrium, upper limits can be set on k and k_- by a comparison of Figure 1 with Figure 3; this comparison gives the values $k_- \leqslant 0.001$ min^{-1} and $k \leqslant 0.0001$ min^{-1} for the *in vitro* systems which were studied.

Discussion

Once the ketone or aldehyde R=O has been unambiguously identified, it should be possible to decide whether A_{Ic} and A_{II} exist independently without interconversion or exist in an enzymically catalyzed or noncatalyzed equilibrium. The system R=O + A_{II} would form a test system for distinguishing these three possibilities. The present experiments give no indication as to whether the blocking group is attached to valine prior to incorporation into the protein or whether it is attached before or after the release of the hemoglobin

from the ribosome. The results of Marchis-Mouren and Lipmann (1965) with hemoglobin F_I, which contains an acetyl group at an N terminus, suggest that the attachment of the acetyl group is independent of protein synthesis and occurs after release of hemoglobin F_{II} from the ribosome.

Our attention has been drawn to the possibility that R=O is attached during the lysis of the cells. This possibility seems unlikely, because the percentage of A_{Ic} that is present in the lysate shows little variation in normal adults; this is true regardless of whether the cells are lysed in a large or small volume of water or whether different methods of lysis are used. If the suggested mechanism were correct, one would expect a mass action effect on the amount of A_{Ic} after lysis under diverse conditions.

In the preceding paper (Holmquist and Schroeder, 1966), it was conjectured that the blocking group in A_{Ic} may be a long chain aliphatic aldehyde or ketone such as stearaldehyde. In this connection it is interesting to think about the possibility that A_{Ic} may be a lipidlike component of the erythrocyte cell wall. If the hemoglobin content is 29 $\mu\mu$g/erythrocyte (Dittmer, 1961) of which 5.3% is A_{Ic}, there are 1.4×10^7 molecules of A_{Ic}/erythrocyte. From the dimensions of the hemoglobin molecule of $50 \times 55 \times 60$ A (Perutz, 1962), a cube of equal volume would have a length of 56 A, and the area of a single face would be 3.1×10^3 A^2. If it is assumed that each A_{Ic} molecule is attached to the cell wall by one face of the equivalent cube, the A_{Ic} in the erythrocyte could cover a total area of $1.4 \times 10^7 \times 3.1 \times 10^3$ A^2 $= 4.5 \times 10^{10}$ A^2. The total surface area of the wet erythrocyte is 163 μ^2 $= 1.6 \times 10^{10}$ A^2 (Dittmer, 1961). Thus the amount of hemoglobin A_{Ic} in the red cell is about that needed to occupy the cell surface. At present, this approximate agreement between the A_{Ic} needed and that present can only be considered an interesting fact which merits further investigation.

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