References and Notes

1. E. T. Mertz, L. S. Bates, O. E. Nelson, *Science* **145**, 279 (1964).
2. E. T. Mertz, N. E. Lloyd, R. Bressani, *Cereal Chem.* **35**, 146 (1958).
3. O. E. Nelson, E. T. Mertz, L. S. Bates, *Science* **150**, 1469 (1965).
4. K. S. McWhirter, *Maize Genet. Coop. News Lett.* **45**, 184 (1971).
5. D. V. Glover, H. M. Barbosa, E. T. Mertz, P. S. Misra, in preparation.
6. R. A. Emerson, G. W. Beadle, A. C. Frazer, *Cornell Univ. Agr. Exp. Sta. Mem. No. 180* (1935).
7. These mutants were grown at Purdue University in 1970. The normal W22 inbred line and the isogenic (o_1) in the same inbred line were grown in Sydney, Australia, in 1970.
8. E. Villegas and E. T. Mertz, *Research Bulletin No. 20* (International Maize and Wheat Improvement Center, CIMMYT, Londres 40, Mexico, D.F., 1971).
9. J. Landry and T. Moureaux, *Bull. Soc. Chim. Biol.* **52**, 1021 (1970).
10. E. T. Mertz, in *Seed Proteins, Synthesis, Properties and Processing*, R. Inglett, Ed. (Avi, Westport, Conn., in press).
11. Paper No. 4609, Purdue Agricultural Experiment Station. Supported by Agency for International Development contract 2809 with Purdue University. We thank M. M. Hassen and L. Tanchoco for technical assistance.

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Biosynthesis of Hemoglobin Ann Arbor: Evidence for Catabolic and Feedback Regulation

Abstract. *Hemoglobin Ann Arbor, in which arginine replaces leucine in position 80 of the α chain, occurs in affected individuals in low proportion to hemoglobin A. Biosynthetic studies were performed on reticulocytes of a patient heterozygous for this hemoglobin. These studies suggested that the low percentage of hemoglobin Ann Arbor is primarily due to preferential destruction of the abnormal component. The reduced concentration of α Ann Arbor chains was also reflected in a decreased synthesis of normal β chains.*

Hemoglobin Ann Arbor is an α chain variant in which the substitution has been shown to be leucine to arginine at position 80 (*1*). The individual in which this variant was originally detected is a Caucasian male who was splenectomized at age 30 because of hemolytic anemia. He now has 12 g of hemoglobin per 100 ml of blood, with 10 percent of red cells as reticulocytes. His erythrocytes are slightly microcytic and moderately hypochromic with moderate anisocytosis and poikilocytosis. Target cells are abundant. Siderocytes are present in the peripheral blood, and the bone marrow is laden with iron. The patient's hemoglobin Ann Arbor component migrates slightly slower than hemoglobin S on starch gel electrophoresis at *p*H 8.6 and comprises about 14 percent of the total hemoglobin.

Hemoglobin Ann Arbor is unstable to heat, a property that frequently disposes hemoglobin to form intraerythrocytic inclusions (*2*). When the erythrocytes of the patient were incubated with crystal violet, a classical pattern of inclusions could not be seen with light microscopy. However, some inclusions suggestive of precipitated hemoglobin have been seen by transmission electron microscopy (*3*).

Abnormal hemoglobins that occur in reduced proportion are not uncommon (*4*). Some hemoglobins are unstable and are known to precipitate readily (*2*). These hemoglobins may be lost from the circulating erythrocytes by the removal of such aggregates of precipitated hemoglobin in the spleen (*5*). A second mechanism, which is supported by experimental evidence, invokes a diminished rate of synthesis (*6*). The patient we used afforded a uniquely desirable combination of circumstances for studying the control of biosynthesis of an abnormal hemoglobin component. Because he lacked a spleen and obvious erythrocyte inclusions, the mechanism of precipitation seemed unlikely. In addition, with 10 percent reticulocytes the patient's blood was expected to be fairly active in the biosynthesis of new hemoglobin. We believed that incubation of samples of this patient's blood in the presence of an isotopically labeled amino acid would indicate whether a diminished rate of synthesis is responsible for the reduced proportion of this abnormal hemoglobin in the peripheral blood or whether some other form of control is manifested.

Venous blood was collected in tubes containing heparin and immediately chilled in ice. Cells were washed four times in NKM (*7*) and suspended in a complete medium, which contained all amino acids required for protein synthesis except leucine, which was added as the ^3H- or ^{14}C-labeled form. Incubation was carried out at 25°C in a Dubnoff metabolic shaking bath. Samples of the incubation were removed at the times shown in Table 1, and further synthesis was stopped by lysis of the cells with four volumes of 1.5 m*M* $MgCl_2$ at 0°C. The ionic strength was rapidly returned to the initial value by addition of a normalizing buffer. The lysates were kept chilled, and the stroma and undisrupted cells were removed by centrifugation at 30,000*g*. Polyribosomes were removed by layering the lysates over cushions of 30 percent sucrose in TKM (*7*) and centrifuging at 100,000*g*. The

Table 1. Specific radioactivities of the purified hemoglobins and their component chains at the time periods indicated. Specific activities (disintegrations per minute per micromole of leucine) and their ratios are given; A and AA refer to hemoglobin A and hemoglobin Ann Arbor, respectively.

Time (min)	Chains							Hemoglobin		
	αA	βA	αA/βA	αAA	βAA	αAA/βAA	αAA/αA	A	AA	AA/A
4	14.2	4.31	3.3	44.1	20.7	2.1	3.1	12.4	32.1	2.6
7	74.2	22.9	3.2	217	103	2.1	2.9	66.5	172	2.6
13	974	286	3.4	2,680	1,220	2.2	2.8	914	2,390	2.5
30	2,740	1,120	2.5	7,420	3,410	2.2	2.7	2,040	5,250	2.6
60	4,840	1,460	3.3	12,600	6,300	2.0	2.6	4,510	11,300	2.5
1,320	38,600	16,300	2.4	100,000	53,800	1.9	2.6	42,700	107,000	2.5
1,320*	1,210	509	2.4	3,150	1,700	1.8	2.6	1,420	3,600	2.5

* Experiment with [^{14}C]leucine.

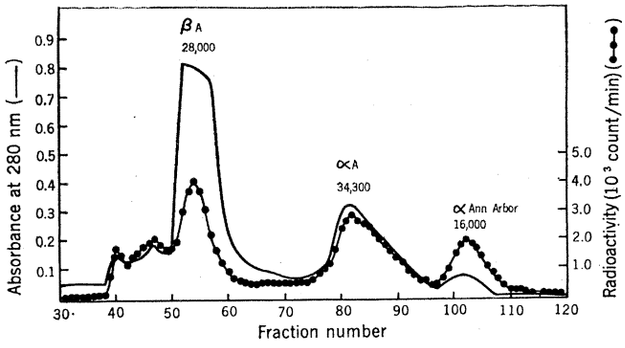

Fig. 1. Chromatography of the whole hemolyzate from the 22-hour [^{3}H]leucine incubation on a CM cellulose column (1 by 10 cm). The column was washed with a solution of 0.005*M* Na_2HPO_4, 0.05*M* 2-mercaptoethanol, and 8*M* urea (*p*H 6.7) until the heme was eluted. At fraction 30 a linear gradient was established from 0.005*M* Na_2HPO_4, 0.05*M* 2-mercaptoethanol, and 8*M* urea (*p*H 6.7) to 0.045*M* Na_2HPO_4, 0.075*M* 2-mercaptoethanol, and 8*M* urea (*p*H 6.7) at a flow rate of 25 ml/hour. Fractions of approximately 4.2 ml were collected, and 0.5-ml samples were counted in Bray's solution containing 2.5 percent thixotropic gel. The dotted line represents radioactivity and the solid line represents absorbance at 280 nm. The numbers above each peak refer to the total counts per minute in that peak.

hemoglobins were separated on diethylaminoethyl (DEAE) Sephadex columns that were equilibrated with 0.05*M* tris buffer, *p*H 8.5, containing 0.001*M* KCN. A decreasing *p*H gradient was used to effect the separation. Both hemoglobin Ann Arbor and hemoglobin A prepared by this method were pure as determined by electrophoresis on cellulose acetate at *p*H 8.6. The specific radioactivities of the hemoglobins were determined by converting the samples into globin by acid acetone precipitation and counting the radioactivity in a liquid scintillation spectrometer. Mass determinations were done by absorbance measurements at 540 nm before conversion into globin. Each [^{3}H]hemoglobin was mixed with a known quantity of [^{14}C]-hemoglobin of the same type with a predetermined specific activity in order to correct for losses during subsequent procedures. [^{14}C]Hemoglobins were obtained by incubation with [^{14}C]leucine for 22 hours at 37°C.

The component chains of the purified hemoglobins were separated on carboxymethyl (CM) cellulose columns according to the procedure of Clegg *et al.* (*8*). The purified α and β chains were desalted by gel filtration on a 90- by 2.5-cm column of Biogel P-2 equilibrated with 0.5 percent formic acid. The radioactivity of these chains was then counted in a liquid scintillation spectrometer. Each sample was internally standardized with [^{3}H]toluene. Mass determinations were done by automatic amino acid analysis for lysine, histidine, and arginine (*9*). All specific activities are expressed as disintegrations per minute per micromole of leucine.

Total radioactivity of the material recovered from each chromatography was calculated and compared with the total that went onto the column. Recoveries were never less than 95 percent, which indicates no significant loss of protein during chromatography.

A sample of each hemolyzate from the incubation of the blood of various family members was placed directly on CM cellulose columns for chain separation without prior purification by DEAE Sephadex chromatography. A typical chain separation of this type is shown in Fig. 1. The radioactivity associated with the α Ann Arbor chains is proportionately much greater than that associated with the αA chains. Furthermore, the total radioactivity of α chains is greater than that of β chains. In contrast, when blood from normal individuals was incubated and chromatographed under identical conditions, the total α chain radioactivity was within ± 2 percent of the total for β chains (*10*). These observations are difficult to reconcile with the supposition that the abnormal hemoglobin is synthesized at a diminished rate. They do, however, suggest that the abnormal hemoglobin may turn over or be catabolized more rapidly than the corresponding normal component. In order to more thoroughly document these observations, the specific activities of the purified hemoglobins and their component chains are shown in Table 1.

The α Ann Arbor chains have about three times the specific activity of the αA chains at all incubation times (Table 1). We believe that these results mean that an increased rate of degradation of the variant chain may be the predominant factor in limiting the proportion of hemoglobin Ann Arbor in the peripheral blood. If α Ann Arbor chains are degraded preferentially, then their concentration would remain lower. It follows that synthesis of both types of α chains at an equal or near equal rate would lead to a higher specific radioactivity of the variant α chains. In contrast, if the lower proportion of variant α chain were due entirely to impaired synthesis, then an equal or reduced specific activity of the variant chain would be expected. The latter model does not fit our results. The hypothesis of preferential degradation is also supported by the results of pulse chase experiments (*10*).

In both hemoglobin A and hemoglobin Ann Arbor the specific activity of the β chain is lower than that of the α chain, and the β chains from hemoglobin Ann Arbor have a higher specific activity than those from hemoglobin A. This result holds even if the material eluting ahead of β chains in some chromatographies (Fig. 1) is assumed to have originated from β chains. Family studies indicate that the patient does not have β thalassemia but does, in fact, have α thalassemia (*11*). Family members with the abnormal hemoglobin and no α thalassemia gene have even lower β chain specific activities relative to those of the α chains. This effect might be due in part to release of unlabeled β chains from the degraded hemoglobin Ann Arbor, with such β chains free to recombine with available α chains. However, the total radioactivity of both types of α chains is almost twice the total for the β chains (Fig. 1). In view of this result it seems more likely that the β chains released by degradation of α Ann Arbor chains suppress the synthesis of new β chains. Similar suppression of rabbit β chain synthesis by added human β chains has been shown in the cell-free system by Shaeffer *et al.* (*12*).

In the normal erythocytes the amount of α chains is precisely balanced by the aggregate of non-α chains (β, γ, and δ). The lack of free hemoglobin polypeptide chains is tacitly accepted as evidence for some form of control mechanism. In this study, the evidence for suppression of β chain synthesis supports this notion. We found that the ratio of total radioactivity in α

chains to that in β chains was almost 2; in contrast, the same ratio in unstable variants of the β chain has been consistently reported to be 1. This difference suggests that the mechanisms governing the rates of synthesis of α and β chains may not be similar. This dissimilarity may explain why there are free α chains present in red cells of unstable β chain variants (*13*), but apparently no free β chains or hemoglobin H in unstable α chain variants.

Although hemoglobin Ann Arbor is preferentially destroyed there are no classical inclusion bodies. This suggests that the degradation may be proteolytic. Moreover, a single amino acid substitution not only reduces the net synthesis of the variant chain, but decreases the synthesis of its complementary subunit, the β chain.

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References and Notes

1. D. L. Rucknagel, N. J. Brandt, H. H. Spencer, in *Genetical, Functional and Physical Studies of Hemoglobin*, T. Arends, G. Bemski, R. Nagel, Eds. (Karger, Basel, 1971), pp. 54–64.
2. H. Lehmann and R. W. Carrell, *Brit. Med. Bull.* **25**, 14 (1969); R. W. Carrell and H. Lehmann, *Semin. Hematol.* **6**, 116 (1969).
3. B. Schnitzer, D. L. Rucknagel, H. H. Spencer, M. Aikawa, *Science* **173**, 251 (1971).
4. H. Lehmann and R. W. Carrell, *Brit. Med. J.* **4**, 748 (1968).
5. R. A. Rifkind, *Blood* **26**, 435 (1965); W. H. Crosby, *ibid.* **12**, 165 (1957); N. S. Lawson, B. Schnitzer, E. B. Smith, *Arch. Pathol.* **87**, 491 (1969).
6. S. H. Boyer, P. Hathway, M. D. Garrick, *Cold Spring Harbor Symp. Quant. Biol.* **20**, 333 (1964); J. M. White and M. C. Brain, *Brit. J. Haematol.* **18**, 195 (1970); G. J. Folayan Esan, F. J. Morgan, J. V. O'Donnell, S. Ford, A. Bank, *J. Clin. Invest.* **49**, 2218 (1970); J. H. Steadman, A. Yates, E. R. Huehns, *Brit. J. Haematol.* **18**, 435 (1970).
7. Abbreviations: NKM, a solution of 0.153*M* NaCl, 0.005*M* KCl, and 0.005*M* $MgCl_2$; TKM, a solution of 0.01*M* tris(hydroxymethyl)-aminomethane (tris) hydrochloride, 1.5 m*M* $MgCl_2$, and 0.05*M* KCl at *p*H 7.4.
8. J. B. Clegg, M. A. Naughton, D. J. Weatherall, *Nature* **219**, 69 (1968).
9. S. H. Boyer, E. F. Crosby, G. L. Fuller, A. N. Noyes, J. G. Adams III, *Ann. N.Y. Acad. Sci.* **165**, 360 (1969).
10. J. G. Adams III, thesis, University of Michigan (1972).
11. ———, W. P. Winter, D. L. Rucknagel, H. H. Spencer, in preparation.
12. J. R. Shaeffer, P. K. Trostle, R. F. Evans, *J. Biol. Chem.* **244**, 4284 (1969).
13. J. M. White and J. V. Dacie, *Nature* **225**, 860 (1970); R. F. Rieder, *J. Clin. Invest.* **50**, 388 (1971).
14. Supported by NIH grants 5-T01-GM-00071-14 and 1P01-GM-15419-05. We thank D. Sweet for technical assistance.

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Somatic Cell Genetic Assignment of Peptidase C and the Rh Linkage Group to Chromosome A-1 in Man

Abstract. *The segregation of the human peptidase-C phenotype in five different series of human-mouse hybrid clones was examined. The chromosome constitution of these hybrids was determined by quinacrine mustard fluorescence, Giemsa banding, and constitutive heterochromatin staining. That the clones could be classified without exception either as human peptidase C positive/A-1 positive (14 clones), or as peptidase C negative/A-1 negative (12 clones) indicates that peptidase C can be assigned to the human A-1 chromosome. Data from an extensive series of human-mouse clones used provide support for the syntenic association between peptidase C and phosphoglucomutase-1 and by inference a linkage of both to Rh factor group.*

We report data that supports the assignment of peptidase C (Pep C), and thus by inference, the rhesus (Rh) linkage group to chromosome A-1 in man. It is possible to establish gene-gene and gene-chromosome linkage relations by the analysis of somatic cell hybrids. Hybrids of human and mouse cells are useful for gene mapping in man because the human chromosomes are preferentially lost, the human phenotypes such as isozymes are constitutively expressed and can be readily detected, and the constitution of human chromosomes can be determined. Linkage relations are determined by correlation. If two phenotypes segregate concordantly (present or absent together in clonal populations of independent origin), then it can be presumed that they are syntenic (situated on the same chromosome). Gene assignment to a particular chromosome can be inferred if a particular human phenotype and chromosome segregate together. Details of the somatic cell genetic approach to linkage analysis have been reviewed (*1*, *2*).

We have used five different combinations of human-mouse hybrids in our studies. The first hybrid has been designated J and resulted from the fusion of mouse RAG cells, which are deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (*3*), with normal human leukocytes (*4*). The cells were selected in the HAT medium (*5*), and derivative subclones were isolated in HAT. The isolation and characterization of the J hybrid clones have been described (*4*). The second hybrid series isolated in HAT

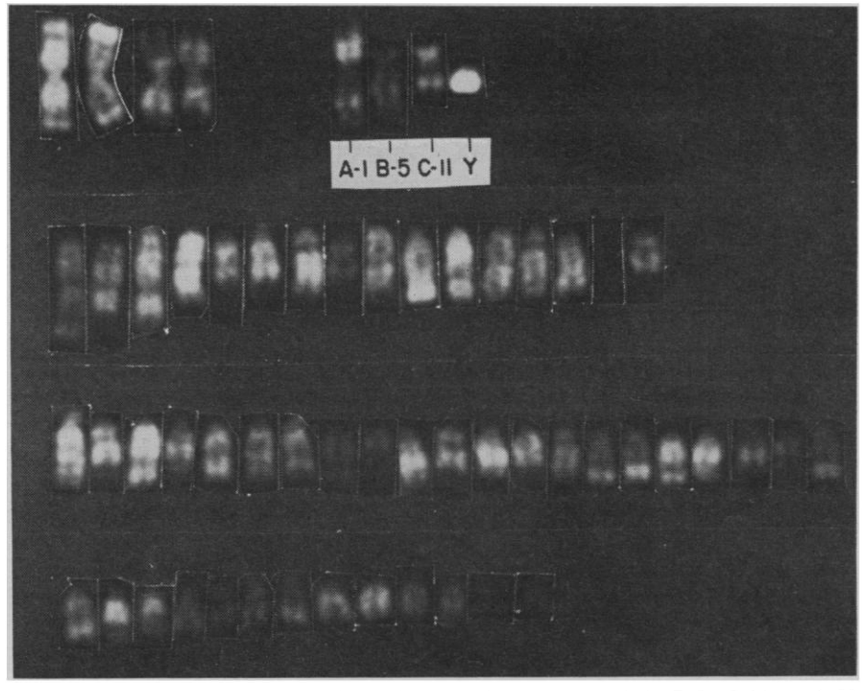

Fig. 1. Human-mouse clone retaining human chromosomes A-1, B-5, C-11, and Y. Unlabeled chromosomes are derived from the mouse RAG parent.