FACTORS INFLUENCING RELATIVE RATES OF SYNTHESIS OF ADULT AND FETAL HEMOGLOBIN *IN VITRO* *

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What causes the gradual decrease of fetal hemoglobin in the normal maturing infant, or the persistence of fetal hemoglobin in the blood of adults with certain congenital anemias, especially thalassemia, is not known. The production of fetal hemoglobin is evidently determined by genes nonallelic with those regulating adult hemoglobin formation (1). The fact that all red cells can be sickled in sickle cell anemia patients having relatively high proportions of fetal hemoglobin indicates that the same cell can produce both an adult hemoglobin (i.e., S) and fetal hemoglobin. This in turn implies that the rate of formation of fetal hemoglobin is not dependent upon the formation of a different cell by a different gene, but reflects a metabolic adaptation by the individual young red cells of the fetus or of the abnormal adult to the cellular environment (2).

The present investigation concerns the effect of cellular environment on the relative quantities of fetal and adult hemoglobin synthesized *in vitro* by the reticulocytes of umbilical cord blood, as determined by the rates of Fe^{59} incorporation into heme and of C^{14}-leucine incorporation into globin.

MATERIALS AND METHODS

Fe^{59} was obtained from Abbott Laboratories, and iron-binding protein of high degree of purity was a gift from Dr. John K. Inman (3). To aqueous solutions of the protein, buffered with 20 mM sodium bicarbonate so as to yield a final pH of 7.4 ± 0.2, sufficient iron either as $Fe^{59}Cl_3$ or $Fe^{56}Cl_3$, was added dropwise to one-third saturate the iron-binding protein. To insure complete protein-binding of the added iron, the mixture was allowed to stand at room temperature for 1 hour prior to its experimental use.

Leucine uniformly labeled with C^{14} was purchased from Nuclear of Chicago and had a specific activity of 43 µc

per mg. Nonradioactive amino acids were obtained from Schwarz Bioresearch Laboratories. IRC-50 resin was supplied by Rohm and Haas Company and purified and equilibrated as previously described (4).

Incubation. From 30 to 50 ml of sterile heparinized umbilical cord blood was obtained at the time of delivery. It was centrifuged at 2,000 G for 30 minutes at 0° C and the top 5 ml of red cells, relatively rich in reticulocytes, was withdrawn and washed twice with Krebs-Ringer phosphate with glucose solution, pH 7.4, at 0° C. In the studies of glucose deficiency, glucose was omitted. An approximately 50 per cent suspension of the washed red cells was made with the same solution and a hematocrit value and reticulocyte count obtained. Two ml aliquots of this red cell suspension were then pipetted into 25 ml siliconized Erlenmeyer flasks. To this was added 1 ml of the amino acid mixtures in Krebs-Ringer phosphate with glucose solution so that their final concentration was twice that of normal fasting levels, as determined by Stein and Moore (5), with the exception of glycine which was made up to 4 times its normal value. In certain experiments valine, isoleucine and histidine were omitted. In other experiments, in which C^{14}-leucine was used as a tracer, the radioactive amino acid was substituted for the stable form.

The red cell suspension was preincubated in a Dubnoff metabolic incubator at 37° C with shaking at approximately 20 times per minute. In experiments on the effects of hypoxia this was done in separate tents under water-saturated gas mixtures containing 95 per cent O_2 and 5 per cent CO_2 or 95 per cent N and 5 per cent CO_2 at a flow rate of 1 L per minute. In other experiments the samples were incubated under water-saturated room air. After 1 hour, there was added sufficient Fe^{59}-labeled iron-binding protein to yield a final concentration of 24 µM and sufficient C^{12}-leucine to yield a final concentration of 2.6 mg per 100 ml. To alternate flasks equal amounts of Fe^{56}-containing iron-binding protein and C^{14}-leucine were added. The flasks were then incubated 4 hours longer.

Separation of hemoglobins. The period of incubation was terminated by the addition of 10 volumes of 0.85 per cent NaCl at 0° C (6) and the red cells were washed 5 times in 0.85 per cent NaCl at 0° C, suspended in 1 ml of 0.85 per cent NaCl and dialyzed overnight against 250 ml of distilled water. The resulting hemolysate was removed from the dialysis sack, 0.8 ml of toluene was added, the solution was shaken vigorously for 5 minutes, after

which 0.4 ml of 5 per cent NaCl was added. The solution was then centrifuged at 2,000 G for 30 minutes or until the middle hemoglobin-containing layer was clear. The hemoglobin was removed by pipetting and dialyzed for 24 hours against a phosphate buffer, 0.06 M in sodium ions, 0.01 M in cyanide ions, pH 6.91 ± 0.02 [Developer no. 4 (4)]. After removal from the dialysis sack, the hemoglobin solutions were found to have a concentration of 70 ± 10 mg per ml. One ml of each sample was then pipetted on a 0.9 × 35 cm column of IRC-50 resin, previously equilibrated against Developer no. 4 and the sample was washed into the column with 0.2 ml samples of developer. The columns were then developed for 5 to 7 hours at 4° C with Developer no. 4 flowing at a rate of 5 to 7 ml per hour (4). By then the main component of adult hemoglobin was 1 to 5 cm from the top of the column, and F_{II}, the main component of fetal hemoglobin (4), was approximately 12 to 30 cm from the top of the column. The resin was removed by extrusion and the individual bands were dissected out and eluted from the resin with 3 ml of saturated disodium phosphate at room temperature. The pH of this eluted hemoglobin solution was 7. The hemoglobin concentration in each band was determined at 540 mμ on a Coleman Junior spectrophotometer. The fetal hemoglobin concentration obtained from the F_{II} band is expressed as the percentage of the total eluted hemoglobin. Since this main component of F hemoglobin includes about 80 per cent of the total fetal hemoglobin (4), the values will be approximately 20 per cent lower than one might expect from alkali denaturation.

Determination of radioactivity. Hemoglobin labeled with Fe^{59} was counted in a well-type scintillation counter. In four experiments, after addition of known amounts of nonradioactive hemoglobin as carrier, the heme was also separated from the globin and crystallized to constant specific activity after the method of Shemin (7). In all instances the value of the activity per milligram of hematin chloride corresponded to that of hemoglobin, assuming heme to constitute 3.9 per cent of hemoglobin. Thus the activity is reported in terms of hemoglobin for ease in comparison with C^{14}-leucine labeling of globin.

In experiments in which C^{14}-leucine was used, the hemoglobin, after elution from the column, was dialyzed against 0.001 M NaCN, the concentration of the hemoglobin was determined, and 0.1 ml samples were pipetted on stainless steel planchets. These samples were then counted with a windowless flow counter, and corrected to a sample size of 0.5 mg using an empirical self-absorption curve. Experiments on large samples demonstrated that all the radioactivity of C^{14}-leucine-labeled hemoglobin, after it had been chromatographically purified, was in the globin fraction and was precipitable by 5 per cent trichloroacetic acid.

RESULTS

Effect of hypoxia. Table I shows the results of six experiments done to investigate the effects of hypoxia on the relative amounts of adult hemoglobin and of fetal hemoglobin synthesized. Since the ratio of fetal/adult hemoglobin is variable from one cord blood sample to another, only comparison with the control ratio is informative. In the last column is given the percentage change in the ratio produced by hypoxia. In every case there

TABLE I

Effect of hypoxia on the relative rates of synthesis of adult and fetal hemoglobin by cord blood reticulocytes in vitro

Label	Reticulo-cytes	F_{II}*	95% O_2			95% N_2			Per cent increase Ratio $\dfrac{FN_2/AN_2 - FO_2/AO_2}{FO_2/AO_2}$
			FO_2	AO_2	Ratio FO_2/AO_2	FN_2	AN_2	Ratio FN_2/AN_2	
	%	%	*cpm/mg Hb*			*cpm/mg Hb*			
Fe^{59}	4.9	57	25	120	0.208	27	97	0.278	34
Fe^{59}	1.1	60	70	201	0.348	130	315	0.413	19
Fe^{59}	6.7	75	43	123	0.350	111	168	0.661	89
			35	87	0.402	66	113	0.584	45
Fe^{59}	15.2	50	1,129	2,270	0.497	613	1,034	0.593	19
			1,117	2,311	0.483	633	1,004	0.630	30
As heme†:									
2nd crystallization			1,424	2,668	0.534	763	1,180	0.647	21
3rd crystallization			1,586	2,947	0.538	858	1,228	0.699	30
Fe^{59}	8.0	59	1,094	2,208	0.495	671	1,142	0.588	19
C^{14}-leucine			1,396	2,630	0.531	1,048	1,785	0.587	11
Fe^{59}	6.0	68	408	866	0.471	863	1,520	0.568	21
C^{14}-leucine			883	1,728	0.511	1,172	2,168	0.541	6

* Main component of fetal hemoglobin determined by chromatography.
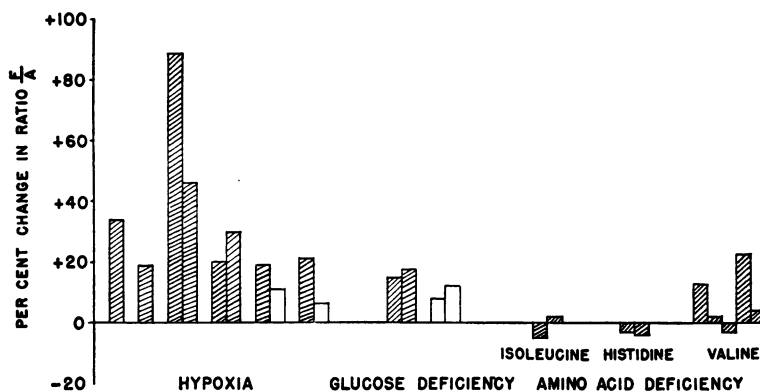† Counted as crystallized heme, but specific activity calculated as hemoglobin.

FIG. 1. EFFECT OF VARIOUS DEFICIENCIES ON THE RELATIVE SYNTHESIS OF
FETAL AND ADULT HEMOGLOBIN BY UMBILICAL CORD RETICULOCYTES *IN VITRO.*
The percentage change in the ratio $\dfrac{\text{fetal hemoglobin synthesized}}{\text{adult hemoglobin synthesized}}$ as com-
pared to a nondeficient control is shown. Bar graphs for experiments in
which Fe^{59} was used as a label are shaded, while those for C^{14}-leucine ex-
periments are unshaded.

was a rise in the ratio of radioactive fetal/adult
hemoglobin, indicating that hypoxia produced a
relative stimulation of synthesis of fetal hemo-
globin compared to adult hemoglobin. A chi-
square test of the ten incubations done here shows
significance at the 0.5 per cent level. Figure 1
summarizes the effects of hypoxia, as well as other
factors, on relative fetal hemoglobin production.

Although hypoxia uniformly stimulated fetal
hemoglobin synthesis relative to adult hemoglobin
synthesis, Table I demonstrates a variable effect
of oxygen deprivation on the specific activity of
hemoglobin (A and F combined). Red blood
cells represent a peculiarly difficult tissue in which
to obtain complete anoxia because of the large
reservoir of oxygen in the hemoglobin. It was
supposed that the variable effect of hypoxia on
the specific activity might be a result of different
degrees of oxygen deprivation achieved, since
there is evidence that with complete anoxia no
hemoglobin synthesis would occur (8). Hence,
in two similar experiments, one of which is il-
lustrated in Figure 2, we departed from the usual
protocol. The Erlenmeyer flasks were stoppered
with airtight vaccine caps and preincubated for
1.5 hours with the following gas mixtures: 95 per
cent O_2 and 5 per cent CO_2; 13 per cent O_2, 82
per cent N_2 and 5 per cent CO_2; and 95 per cent
N_2 and 5 per cent CO_2. In one flask incubated
with 95 per cent N_2 and 5 per cent CO_2, a well
containing 0.5 ml of 35 per cent hydrosulfite was

employed to further decrease the oxygen tension.
The Fe^{59}-iron-binding protein was then added
under nitrogen, and the sealed flasks incubated
for 5.5 hours longer. The percentage saturation
of each hemoglobin sample was then determined
by the method of Gordy and Drabkin (9). The
treatment of the samples and the crystallization of
the heme to constant specific activity were then
carried out as usual. It can be seen from Figure
2 that mild hypoxia tended to increase the hemo-
globin specific activity. This increase in specific
activity may not reflect increased hemoglobin
synthesis since, for example, hypoxia may cause
a shrinkage of the intracellular pool of nonlabeled
iron during preincubation, with a consequent
lesser dilution of the radioactive label. The con-
sistently greater relative stimulation of fetal he-
moglobin synthesis under conditions of severe
hypoxia is evident again.

An attempt was made to determine the time
course of the effects of hypoxia on fetal and adult
hemoglobin synthesis. Equal portions of the
reticulocyte-rich umbilical cord red cells were in-
cubated in four separate Erlenmeyer flasks with
Fe^{59}-labeled iron-binding protein as usual, two
under 95 per cent O_2 and 5 per cent CO_2, and two
under 95 per cent N_2 and 5 per cent CO_2. After
two hours the incubation was stopped for a pair
of flasks, one oxygenated and one hypoxic, while
the remaining pair was incubated for four hours
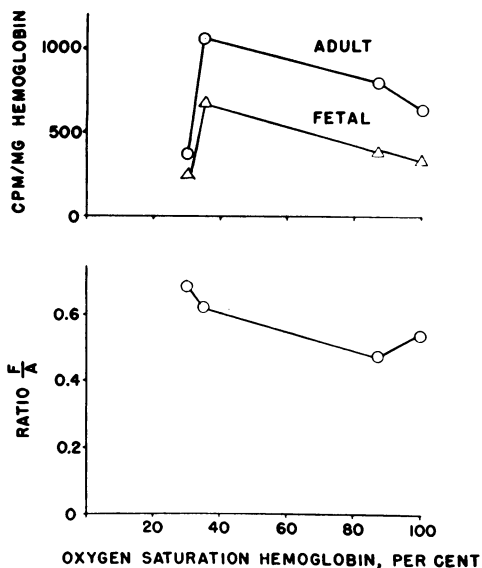as usual. The result of this experiment is shown

FIG. 2. EFFECT OF VARIOUS DEGREES OF OXYGEN DE-
FICIENCY ON THE SYNTHESIS OF ADULT AND FETAL HEMO-
GLOBIN *IN VITRO*. The changes in specific activity of
the adult and fetal hemoglobins recovered are depicted
in the top portion of the figure; the ratio of fetal hemo-
globin specific activity to adult hemoglobin specific ac-
tivity is shown in the bottom portion.

in Figure 3. A significant change in the ratio of
hemoglobin synthesized did not occur until the
last two hours of the four hour incubation. This
was due apparently to a relatively greater decrease
in the rate of fetal hemoglobin production in the
95 per cent O_2 environment.

Effect of metabolic inhibition. The effect of
glucose deficiency, cyanide and methylene blue
may be seen in Table II. Glucose deficiency, like
oxygen lack, depressed adult hemoglobin synthe-
sis relatively more than fetal hemoglobin synthesis.
The addition of 120 mg per 100 ml desoxyglucose
to the glucose-deficient medium had no additional
effect. Both cyanide and methylene blue almost
completely stopped synthesis of both hemoglobins,
so that the ratios are of little quantitative signifi-
cance, but are included to demonstrate that inhi-
bition of these types did not favor fetal hemoglobin
synthesis.

Effect of amino acid deficiencies. The effect of
various amino acid deficiencies may be seen in
Table III. Fetal and adult human hemoglobin
differ in valine, isoleucine, and histidine content.

Fetal hemoglobin has less valine than adult he-
moglobin has (9.5 vs 11.2 g per 100 g), the miss-
ing valine being replaced almost mole for mole by
isoleucine (1.46 g per 100 ml), which the main
component of adult hemoglobin lacks entirely (4,
10). Fetal hemoglobin also has less histidine
(7.7 vs 8.4 g histidine per 100 g) (10). Thus,
the effects of deficiencies of these three amino
acids were studied. From Table III we see that
isoleucine, and histidine deficiencies in the incu-
bation medium had no effect on Fe uptake. Va-
line deficiency in two experiments seemed to in-
crease relative fetal hemoglobin synthesis and al-
though the results here are not significant, they
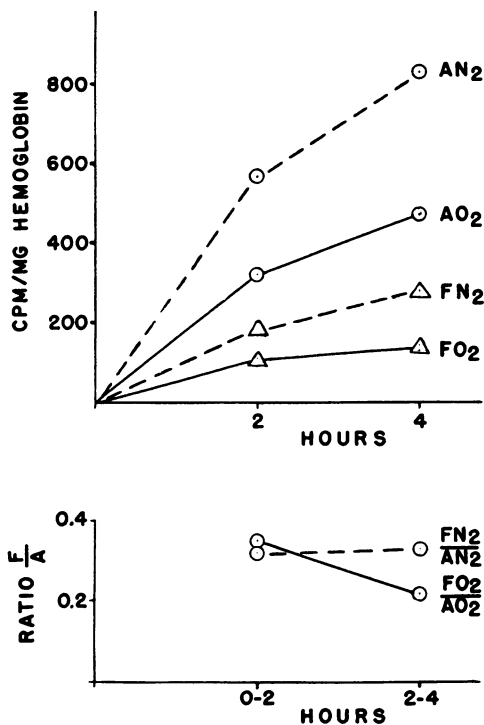suggest further study.

FIG. 3. TIME COURSE OF SYNTHESIS OF FETAL AND
ADULT HEMOGLOBIN BY UMBILICAL CORD RETICULOCYTES
UNDER NITROGEN AND UNDER OXYGEN *IN VITRO*. The
rate of synthesis of both A hemoglobin (○) and F he-
moglobin (△) diminished during the second two-hour
period of incubation both under oxygen (———) and un-
der nitrogen (– – –). Diminution in hemoglobin syn-
thesis was greatest in respect to the rate of F hemo-
globin synthesis under oxygen. In the lower diagram
the ratio of fetal to adult hemoglobin synthesized is
compared for the first two-hour period with the ratio
for the second two-hour period.

TABLE II

Effect of glucose deficiency, cyanide, and methylene blue on the relative synthesis of adult and fetal hemoglobin by cord blood reticulocytes in vitro

Label	Reticulo-cytes	F_{II}*	Addition to flask		F	A	Ratio F/A	Per cent change from control ratio F/A in inhibited flask
	%	%			*cpm/mg Hb*			
Fe^{59}	2.3	56	Control	Glucose	137	332	0.41	
			Inhibited	0	149	319	0.47	15
					138	290	0.48	17
C^{14}-leucine	4.5	66	Control	Glucose	603	1,154	0.52	
					577	1,160	0.50	
			Inhibited	120 mg % desoxy-glucose	646	1,154	0.56	8
					645	1,157	0.56	12
			Inhibited	Glucose + 10^{-2} NaCN	31	91	0.34	−33
					24	91	0.26	−49
			Inhibited	Glucose + 0.002% methylene blue	28	86	0.33	−35
					35	109	0.32	−37

* Main component of fetal hemoglobin determined by chromatography.

DISCUSSION

Hypoxia uniformly favored the production of fetal hemoglobin compared with adult hemoglobin as seen in Table I and Figure 1. In the presentation of these experiments, we have emphasized the *ratio* of fetal and adult hemoglobin synthesized rather than their individual rates of synthesis. Since both fetal and adult hemoglobin-synthesizing mechanisms apparently share the same pool, this ratio of the specific activity of two hemoglobins, made in the same cell, is presumably not influenced by changes in the intracellular pool size. Although, as mentioned earlier, both F and A hemoglobin are probably synthesized within the same cells, there is some evidence that the proportions of adult and fetal hemoglobins may vary somewhat from one cell to another (11). Thus it is possible that the adult and fetal synthesizing

mechanisms are present in young red cells in varying amounts. This should not, however, invalidate the measurement of the ratio of hemoglobins synthesized.

The generally accepted explanation of the function of fetal hemoglobin is that it represents a specific adaptation for life *in utero,* by providing the fetus with red cells with a greater affinity for oxygen (12). While human fetal red cells do indeed have a greater affinity for oxygen at a given Po_2 than do adult red cells (i.e., the oxygen dissociation curve is "shifted to the left") (12, 13), the curve for hemolyzed fetal red cells is actually to the right of that of a hemolysate of maternal red cells, and when both hemoglobins have been dialyzed their oxygen dissociation curves become identical (14). Furthermore, whereas fetal red cells may lose their heightened oxygen affinity within a month after birth (12), fetal hemoglobin

TABLE III

Effect of deficiency of various amino acids on rates of adult and fetal hemoblogin synthesis in vitro by cord blood reticulocytes, using Fe^{59} incorporation into hemoglobin

Reticulo-cytes	F_{II}*	Complete			−Isoleucine			Per cent change $\frac{F_I/A_I - F_c/A_c}{F_c/A_c}$	Deficient −Histidine			Per cent change $\frac{F_I/A_I - F_c/A_c}{F_c/A_c}$	−Valine			Per cent change $\frac{F_I/A_I - F_c/A_c}{F_c/A_c}$
		F_c	A_c	Ratio	F_I	A_I	Ratio F_I/A_I		F_I	A_I	Ratio F_I/A_I		F_I	A_I	Ratio F_I/A_I	
%	%	*cpm/mg Hb*			*cpm/mg Hb*				*cpm/mg Hb*				*cpm/mg Hb*			
5.6	46	147	369	0.40	144	380	0.38	−5					105	233	0.45	13
4.3	61	196	410	0.48	182	375	0.49	2					146	300	0.49	2
4.6	74	120	346	0.35					115	340	0.34	−3	110	321	0.34	−3
2.4	66	61	129	0.47					59	131	0.45	−4	46	80	0.58	23
													40	82	0.49	4

* Main component of fetal hemoglobin determined by chromatography.

disappears at a more leisurely pace (15). Thus, the concept that the primary function of fetal hemoglobin is for its specific properties in oxygen transport is difficult to defend. However, the possibility that the increased affinity of fetal hemoglobin for oxygen within the red cell is due to a property of fetal hemoglobin not evident in solution, has not been ruled out.

An alternate explanation is suggested by the consideration that the organism producing fetal hemoglobin has many other demands for oxygen, energy-yielding metabolites, and essential amino acids. Hence, a protein with a smaller content of certain essential amino acids and requiring less energy to produce might possess an advantage for the fetus.

The importance of hypoxia, glucose deficiency, or possibly valine deficiency in directly regulating fetal hemoglobin production can only be suggested, not determined, by these *in vitro* experiments. At birth the blood flowing to the fetus from the placenta, before respiration, has usually been found to be about 50 per cent saturated with oxygen (16). Certainly one might expect this degree of saturation to be further reduced in the tissue capillaries. The fetus seems to be well supplied with glucose, although the possibility of local deficiency cannot be excluded. Valine deficiency is evidently not severe in the fetus (17) but local tissue deficiency may exist. Although the changes in synthesis rates reported here are small, it must be remembered that the incubation period was carried out during only a small segment of the total time of hemoglobin synthesis by the maturing red cell. Therefore, an effect of one or all of these deficiencies in the direct regulation of fetal hemoglobin production seems possible.

The observation that time of birth during fetal life has no discernible effect on the rate of replacement of fetal hemoglobin (18, 19) is against any direct mediation of oxygen on the regulation of fetal hemoglobin production. Since rapid growth rate, rather than intrauterine existence, might condition local glucose or valine deficiency, this same observation is less opposed to the idea of these substances as regulators.

Regardless of the role of these deficiencies in the regulation of fetal hemoglobin production in the normal infant, it is possible that fetal hemoglobin is an adaptation to these deficiencies. This

possibility provides impetus for the investigation of defects of energy or amino acid metabolism in congenital anemias of adults characterized by large amounts of fetal hemoglobn, such as thalassemia.

SUMMARY

1. Reticulocyte-rich suspensions of umbilical cord blood red cells were incubated *in vitro* with Fe^{59}-iron-binding protein, or C^{14}-leucine, and the amounts of Fe^{59}-labeled or C^{14}-labeled fetal or adult hemoglobin newly synthesized were measured separately.

2. Conditions of hypoxia, or glucose deficiency, favored the formation of fetal hemoglobin as compared to adult hemoglobin.

3. It is possible that fetal hemoglobin may be an adaptation to the fetal environment in which oxygen and glucose are at a premium.

ADDENDUM

Since this paper was submitted for publication a communication presenting similar observations has appeared [Thomas, E. D., Lochte, H. L., Jr., Greenough, W. B., II., and Wales, M. *In vitro* synthesis of foetal and adult haemoglobin in foetal haematopoietic tissues. Nature (Lond.) 1960, **185**, 396]. This finding of an increase in the proportion of hemoglobin F synthesis relative to hemoglobin A with anoxia, is in agreement with our results despite differences in tissue used, length of incubation, radioactive label, and method of isolation of the hemoglobins.

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