Effect of Actinomycin D, Cycloheximide, and Acute Blood Loss on Ferritin Synthesis in Rat Liver

By J. A. MILLAR, R. L. C. CUMMING, JACQUELINE A. SMITH AND A. GOLDBERG

Medical Research Council Group in Iron and Porphyrin Metabolism, University Department of Medicine, Western Infirmary, Glasgow W.1, U.K.

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1. The mechanism of the stimulation of ferritin synthesis by iron *in vivo* has been studied in rat liver. Ferritin synthesis and turnover was measured by [^{14}C]leucine incorporation. 2. Actinomycin D had no inhibitory effect, after administration of iron, on [^{14}C]leucine incorporation into ferritin but appeared to augment the effect of iron on ferritin synthesis. 3. Cycloheximide completely abolished the stimulation by iron of [^{14}C]leucine into ferritin and was subsequently utilized to show that iron acts *in vivo* by translational induction of apoferritin synthesis, rather than by stabilization of apoferritin or its precursors. 4. This conclusion was confirmed by showing that 2 days after acute bleeding, when iron was in the process of being removed from hepatic ferritin stores, ferritin synthesis was decreased whereas breakdown rates were unchanged.

Ferritin, an iron-storage protein of widespread occurrence, was discovered in horse spleen by Lauffer (1937). Granick (1946) showed histologically that the content of ferritin in the intestinal mucosa of guinea pigs increased if iron was added to the diet, and postulated that iron acted by stabilization of apoferritin molecules. The ferritin content of rat liver after oral and parenteral administration of iron was studied by Fineberg & Greenberg (1955), who suggested an alternative hypothesis, namely that iron caused synthesis of ferritin *de novo*. Granick's (1946) original suggestion, that iron acted by stabilization of apoferritin, was revived by Drysdale & Munro (1966). More recently, however, these authors have appeared to favour a mechanism based on induction of synthesis *de novo* (Drysdale, Olafsdottir & Munro, 1968).

There has also been controversy about the possibility that iron acts by activation or derepression of the gene responsible for apoferritin biosynthesis. Drysdale & Munro (1966) found that actinomycin D did not inhibit the increase in rat liver ferritin synthesis after iron administration, and similar results were obtained in HeLa cells by Chu & Fineberg (1969). These authors concluded that the control mechanism is confined to the cytoplasm of the cell. Other studies, however, suggested that actinomycin administration did lead to an inhibition of ferritin synthesis (Yu & Fineberg, 1965; Yoshino, Manis & Schachter, 1966, 1968), and it was concluded that a necessary preliminary to

increased ferritin synthesis was the synthesis of new mRNA.

The present study was undertaken to ascertain whether the mechanism by which iron stimulates ferritin synthesis involves participation of the gene, and if not whether iron acts by induction at the level of translation or by stabilization of apoferritin or its precursors. Actinomycin D, which blocks DNA-dependent synthesis of RNA (Reich, Franklin, Slotkin & Tatum, 1961) was used to test for genetic involvement in ferritin synthesis, and cycloheximide, which inhibits protein synthesis in rat liver (Yeh & Shils, 1969) was used to distinguish between induction and stabilization. The results suggest that iron acts solely by causing synthesis of ferritin *de novo*, and not at all by stabilization.

Further evidence that this is the case was obtained by measuring ferritin turnover in rats while iron was being mobilized from hepatic ferritin stores as a result of acute bleeding 2 days previously.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Animal Suppliers Ltd., Welwyn, Herts., U.K.) (body wt. 250 ± 10 g) were used throughout this study, except where indicated. Actinomycin D was supplied by Merck, Sharp and Dohme Ltd., Hoddesdon, Herts., U.K.; cycloheximide was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; [^{14}C]leucine (55.2 mCi/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Iron

was administered as iron sorbitol citrate ('Jectofer') manufactured by Astra-Hewlett Ltd., Watford, Herts., U.K. Horse spleen ferritin was supplied by Mann Research Laboratories, New York, N.Y., U.S.A., and was further purified by downward elution through Sephadex G-200 before use. All other reagents and chemicals were AnalaR grade or equivalent.

Timing of experiments. The time-intervals used in the animal experiments were based on two considerations. First, when iron is administered to rats, maximal incorporation of pulse doses of [^{14}C]leucine into ferritin occurs after approx. 5h. Secondly, the incorporation of [^{14}C]leucine into ferritin is itself complete after 2h (Drysedale & Munro, 1966). The radioisotope was therefore administered 3h after the animals had been given iron and 2h before death. The dose of [^{14}C]leucine used throughout was 5 $\mu\text{Ci}/100\text{g}$ body weight.

Extraction of ferritin. At the end of each experiment the rats were killed by a sharp blow to the head. The livers were washed with ice-cold 0.9% NaCl, then homogenized in deionized water with an MSE homogenizer, so that the final concentration of tissue was 20% (w/v). This concentration gives optimum ionic strength for precipitation of mixed liver protein during the subsequent step, in which the homogenate was heated to 80°C (Drysedale & Ramsay, 1965). At this temperature ferritin is resistant to denaturation and remains in solution (Laufberger, 1937). The heat-coagulated tissue proteins were removed by centrifugation at 0°C (1400g) but if the supernatant fractions were cloudy at this stage they were clarified by centrifugation at 15000g.

Immunological isolation of ferritin. Ferritin was isolated immunologically from a 2ml sample of the clear 'heat-supernatant' fraction by precipitation with rabbit anti-(horse spleen ferritin). This antibody is known to cross-react with rat ferritin (Mazur, Grien & Carleton, 1960) and since the antigen-antibody ratio is constant if the conditions of precipitation are strictly controlled and uniform (Mazur & Shorr, 1950a) immunological precipitation of ferritin may be used analytically. To each 2ml sample of liver 'heat-supernatant' fraction at 37°C was added a standardized volume of antiserum. Standardization was accomplished by precipitating ferritin from several 2ml samples of one liver 'heat-supernatant' fraction with graded volumes of antiserum from 0.05 to 1ml. The volume of antiserum chosen was that which just precipitated all the ferritin in the 2ml sample, and was normally 0.4–0.6ml of serum. The mixture was incubated for 1h at 37°C, then transferred to a cold-room at 2°C for 12h. After centrifugation (900g) the precipitate was washed twice with ice-cold 0.9% NaCl and the residue was dissolved in 0.1ml of 0.1M-NaOH and made up to 10ml with deionized water.

Analytical methods. Ferritin protein was measured on two 1ml samples of the 10ml solution by the method of Lowry, Rosebrough, Farr & Randall (1951) by using a standard graph calibrated with rat apoferritin. To the 8ml of solution remaining after protein analysis was added 2ml of 1.65M-HClO₄. The solutions were left overnight at 0°C and the precipitated protein was removed by centrifugation (900g). After being washed twice with 0.35M-HClO₄ the precipitates were counted for radioactivity. In the actinomycin D experiments the samples were dissolved in 0.1ml of 0.1M-NaOH and placed on

washed stainless-steel planchets lined with tissue paper to standardize self-absorption (Garrow & Piper, 1955). The samples were counted on a Nuclear-Chicago gas-flow-type counter to a standard error of $\pm 3\%$. In all other experiments the protein precipitate was dissolved in a solution of Hyamine hydroxide 10% (v/v) in 50% (v/v) methanol to which was added 10ml of dioxan scintillator (Bray, 1960). The samples were counted on a Packard Tri-Carb spectrometer. Readings were corrected to 100% efficiency by using ^{14}C -quenched standards supplied by Packard Instrument Co. Inc. (La Grange, Ill., U.S.A.). The reproducibility of the method was measured by precipitating ferritin from six samples of one liver 'heat-supernatant' fraction as above, and analysing for both protein and radioactivity. The value obtained was $\pm 3\%$.

Iron was determined by a modification of the method of Hill (1931). Proteinous material was digested with 18M-H₂SO₄ and 16M-HNO₃. After removal of excess of HNO₃ by boiling, 2ml of 5M-HCl was added to form soluble ferric chloride. The mixture was left overnight and filtered. The pH of the filtrate was adjusted to 3 with NH₃ (sp.gr. 0.88) and 6M-H₂SO₄, with *p*-nitrophenol [1% (w/v) in 10% (v/v) ethanol] as indicator. To the mixture, contained in a 20ml volumetric flask, was added 1ml of 2% (w/v) 2,2'-bipyridyl, 1ml of 60% (v/v) acetic acid, 1ml of 1.75M-Na₂SO₃ as reducing agent, and water to 20ml. A 10ml sample was heated to 100°C for 15min. Any decrease in volume was made good with water. The E_{520} was measured spectrophotometrically (Unicam SP.500) and converted into μg of iron by comparison with standards. All glassware used in iron determinations was rendered iron-free by immersion in 50% (v/v) HCl for 24h.

Studies with actinomycin D. Actinomycin D was administered intraperitoneally into rats (wt. 160 $\pm$ 5g) at two doses, namely 100 and 150 μg of actinomycin/100g body wt, as a solution in sterile 0.9% NaCl. These doses have been shown to cause an 80% inhibition of [^{14}C]adenine incorporation into rat liver RNA without decreasing the incorporation of radioactive amino acids into mixed liver protein (Drysedale & Munro, 1966). Thus the doses used in the present study were not sufficiently high to cause a non-specific decrease in protein synthesis during the time-period of the experiment.

Studies with cycloheximide. Cycloheximide was administered intraperitoneally as a solution in 0.02M-tris-HCl buffer, pH 7.4. This buffer had no effect on the incorporation of [^{14}C]leucine into rat liver ferritin. The solution was made up immediately before use to minimize any possible hydrolysis of the β -hydroxy ketone group. The dose administered, 1 μg of cycloheximide/g body wt, is known to cause a 95% inhibition of amino acid incorporation into mixed liver protein, the effect being greatest 4–6h after administration of the antibiotic (Yeh & Shils, 1969).

Cycloheximide was used in two experiments. The first of these measured its effect on ferritin synthesis, and in the second experiment the drug was used to distinguish between stabilization of ferritin and induction of *de novo* synthesis (see the Results section).

Ferritin synthesis after acute bleeding. To discover the period that elapses between acute bleeding and the decrease in hepatic iron content caused by this treatment (Granick, 1943; Shoden, Gabrio & Finch, 1953) rats were

bled through a cardiac puncture under light ether anaesthesia. Whole blood (5ml) was withdrawn from rats, which were killed at daily intervals thereafter in groups of four. Liver ferritin and haemosiderin were fractionated as follows. Haemosiderin, being insoluble in water, was precipitated with mixed liver protein when 5ml of a 20% (w/v) liver homogenate was heated to 80°C. Ferritin, on the other hand, remained in solution and was recovered from the supernatant fraction by addition of an equal volume of 5.2M-(NH₄)₂SO₄ (Fulton & Ramsay, 1960). The iron content of the washed coagulum can be assumed to be derived from haemosiderin only, unless iron stores are very low (Hampton & Kahn, 1953). Iron remaining in solution after precipitation of ferritin was designated 'non-storage' iron, and comprises mainly iron in haemoglobin and other haem pigments (Drysdale & Ramsay, 1965). The iron content of each fraction, as well as the total iron in an unfractionated 1ml sample of the liver homogenate, was determined as described above.

RESULTS

Effect of actinomycin D. Six groups of rats were treated as shown in Table 1. The time-intervals between actinomycin D and iron administration were zero, 2 and 3 h in groups III, IV and V respectively. Since there was no significant difference in the results at each dose of actinomycin, only those for the upper dose (150 µg of actinomycin/100g body wt.) are given (Table 2). The iron-induced stimulation of [¹⁴C]leucine incorporation into ferritin is shown by the higher value of specific radioactivity in group II, which received iron, compared with that of group I which did not ($P < 0.001$, Student's 't' test). In the group of rats that received actinomycin, no inhibition of this effect is apparent. Moreover, as the interval between administration

Table 1. *Experimental programme for actinomycin D experiments*

Compounds were administered as follows: actinomycin D, intraperitoneally as a solution in sterile saline, 150 µg/100g body weight; iron, intramuscularly as iron sorbitol citrate ('Jectofer'), 1 mg/100g body weight; [¹⁴C]leucine, intraperitoneally in sterile saline, 5 µCi/100g body weight. Abbreviation: Act D, actinomycin D.

Group	Time (h)								No. of animals	
	0	1	2	3	4	5	6	7		8
I		Saline			[¹⁴ C]-leucine		Death			12
II		Iron			[¹⁴ C]-leucine		Death			14
III	Iron + Act D			[¹⁴ C]-leucine		Death				20
IV	Act D		Iron		[¹⁴ C]-leucine			Death		15
V	Act D			Iron			[¹⁴ C]-leucine		Death	12
VI	Act D			Saline			[¹⁴ C]-leucine		Death	12

Table 2. *Effect of actinomycin D on [¹⁴C]leucine incorporation into rat liver ferritin and on the stimulation of [¹⁴C]incorporation caused by iron*

Results are given as mean $\pm$ S.E.M. The key to groups I–VI is given in Table 1. Statistics show the significance of the increases in total radioactivity (c.p.m.) in groups II–VI over that in group I (Student's 't' test). The difference in total radioactivity between groups III and V, which differed only in the time-interval between administration of iron and actinomycin D, is also significant ($P < 0.01$).

Group	Ferritin protein (mg/liver)	Total radioactivity (c.p.m./liver)	Specific radioactivity (c.p.m./mg)
I	5.00 $\pm$ 0.75	175 $\pm$ 33	35 $\pm$ 13 —
II	4.93 $\pm$ 0.49	688 $\pm$ 82	134 $\pm$ 35 $P < 0.001$
III	4.48 $\pm$ 0.61	683 $\pm$ 91	154 $\pm$ 40 $P < 0.001$
IV	7.45 $\pm$ 2.24	1098 $\pm$ 269	148 $\pm$ 65 $P < 0.001$
V	12.52 $\pm$ 3.21	1929 $\pm$ 457	156 $\pm$ 52 $P < 0.001$
VI	6.40 $\pm$ 2.21	417 $\pm$ 59	66 $\pm$ 15 $P < 0.01$

of actinomycin and iron increased from zero to 3h, the value of [^{14}C]leucine incorporation also increased (Table 2).

Effect of cycloheximide. The inhibition of ^{14}C -labelled amino acid incorporation into rat liver protein is maximal 4–5h after administration of cycloheximide (Yeh & Shils, 1969). The time-interval between administration of iron and the peak rate of ferritin synthesis that it stimulates is of the order of 5h (Drysedale & Munro, 1966). Cycloheximide was therefore administered 30min before iron so that these two maxima should coincide.

The effect of cycloheximide on ferritin synthesis was measured in three groups of rats. Group I received both iron and cycloheximide. Group II was given iron and 0.5ml of 0.9% sodium chloride in place of cycloheximide, and group III, which was given neither iron nor cycloheximide, served as the normal control. Each group was also given [^{14}C]leucine. The mean specific radioactivities of ferritin (c.p.m./mg) in groups I, II and III were 12, 185 and 79 respectively. Cycloheximide thus produced a 95–100% inhibition of the increase in [^{14}C]leucine incorporation into ferritin caused by the administration of iron. As expected from other studies, there was a similar decreased incorporation of [^{14}C]leucine into mixed liver protein (Young, Robinson & Sacktor, 1963; Trakatellis, Montjar & Axelrod, 1965).

Cycloheximide was subsequently utilized in an experiment designed to determine whether iron can stabilize apoferritin against breakdown or whether it acts by causing synthesis *de novo*. Five groups of rats were treated as shown in Table 3. [^{14}C]Leucine was administered to each group. After 2h the quantity of free radioactive leucine in the amino acid pool is negligible, and no further labelling of ferritin should occur. Nevertheless, because of the importance of uniform labelling in each group, a 3h period was allowed to elapse before further treatment. Since the half-life of labelled

ferritin *in vivo* is at least 72h (Drysedale & Munro, 1966) the loss of activity over the added 1h is negligible. Group I rats were killed 3h after leucine administration. The value of ferritin radioactivity in this group was taken as a measure of the radioactivity of ferritin in groups IV and V at the time of cycloheximide administration.

Since cycloheximide abolishes ferritin synthesis the interest of this experiment lies in the effect of iron on the breakdown of ferritin in groups II–V. Of these, two received iron (groups III and V) and two received cycloheximide (groups IV and V). The results are shown in Table 3. The activity of ferritin 3h after [^{14}C]leucine administration, given by group I, was 200 c.p.m./mg. In group IV, which received cycloheximide, continuing ferritin breakdown in the absence of synthesis, led to significantly decreased ferritin protein content and ferritin radioactivity ($P < 0.01$, Table 3). Thus the specific radioactivity of ferritin did not change significantly from the value at the time of cycloheximide administration. Iron, which was administered as well as cycloheximide to group V, had no effect on this pattern of breakdown (Table 3) and thus did not appear to have a stabilizing effect on ferritin.

Comparison of groups II and III confirms that iron does not act by stabilization of pre-existing ferritin. Cycloheximide was not administered to the rats in those two groups and injection of iron caused an increase in ferritin, as witnessed by the significantly higher values for ferritin protein in that group ($P < 0.001$). This increase, however, cannot be due to an accumulation of 'stabilized' ferritin, because the total radioactivities (c.p.m.) in groups II and III are similar. If iron acted by stabilization of ferritin a higher value for total radioactivity would be expected in the group that received iron.

Ferritin synthesis after acute bleeding. Removal of 5ml of whole blood from rats (equivalent to 2–2.5mg of iron) by cardiac puncture was found to cause a decrease in total hepatic iron content after

Table 3. *Effect of cycloheximide on the radioactivity of ferritin in rat liver*

Rats were given [^{14}C]leucine (5 $\mu\text{Ci}/100\text{g}$) 3h before iron and 3.5h before cycloheximide, as indicated by +. The rats in groups I and II were killed 3 and 8h after [^{14}C]leucine administration respectively. Groups III–V were killed 8h after [^{14}C]leucine administration.

Group	I	II	III	IV	V
No. of animals	20	20	20	21	19
Cycloheximide (1 $\mu\text{g}/\text{g}$)	—	—	—	+	+
Iron (1 mg/100g)	—	—	+	—	+
Ferritin protein (mg/liver)	4.97 $\pm$ 0.86	4.90 $\pm$ 0.97	9.56 $\pm$ 2.62	3.95 $\pm$ 0.83	3.90 $\pm$ 0.78
Ferritin radioactivity (c.p.m./liver)	996 $\pm$ 167	895 $\pm$ 106	905 $\pm$ 92	815 $\pm$ 125	830 $\pm$ 107
Specific radioactivity (c.p.m./mg)	200 $\pm$ 63	183 $\pm$ 79	95 $\pm$ 31	204 $\pm$ 72	210 $\pm$ 54

a lag period of 24h (Fig. 1). The decrease was compounded from decreases in both haemosiderin and ferritin iron content, although the loss of iron from ferritin was greater. 'Non-storage' iron remained constant throughout the experiment.

In rats, bleeding also results in a depression of hepatic ferritin content (Cumming, Smith, Millar & Goldberg, 1970). This result could be produced *in vivo* either by decreasing the rate of ferritin synthesis or by increasing the rate of degradation. To distinguish between these two possibilities, the effect of removing iron from stores (by acute blood loss) on the total ferritin radioactivity 2 and 72h

after administration of [¹⁴C]leucine was measured. [¹⁴C]Leucine was administered on the second day after acute blood loss. The radioactivity after 2h represents the balance of synthesis and breakdown and is not a meaningful measure of the rate of either process. However, the loss in total ferritin radioactivity between 2 and 72h depends only on the rate of breakdown, since after 2h no further labelling of ferritin occurs.

Twelve rats bled through a cardiac puncture (5ml) were administered with [¹⁴C]leucine 2 days later. Of this group six were killed after 2h, but 72h elapsed before the remaining six were killed. The 72h period was chosen because it has been reported as the half-life of labelled ferritin *in vivo* (Drysdale & Munro, 1966). Liver ferritin radioactivity was assayed as described in the Materials and Methods section. The radioactivity of ferritin in normal rats 2 and 72h after administration of [¹⁴C]leucine was also measured. The results are shown in Table 4. Values for both total radioactivity (c.p.m.) and specific radioactivity (c.p.m./mg) 2h after leucine administration were significantly decreased in the bled rats (*P*<0.01). This suggests that the ferritin content tends to decrease as soon as the iron content of the liver decreases. There was, however, no significant difference in the loss of ferritin radioactivity between the normal and the bled rats over 72h.

Fig. 1. Rat liver iron content after acute blood loss by cardiac puncture (5ml) at time zero. Liver iron was fractionated into four components as explained in the Materials and Methods section. Each point represents the mean of four analyses. ●, Total hepatic iron (mg); △, haemosiderin iron; ▲, 'non-storage' iron; ○, ferritin iron.

DISCUSSION

In mammals ferritin acts as a vehicle for storing iron, as a means of regulating iron absorption (Smith, Drysdale, Goldberg & Munro, 1968), and as a convenient method of protection against the toxic effects of free iron. The ability of ferritin to perform these and other functions derives from the fact that intracellular concentrations of ferritin respond with sensitivity to the presence or absence of iron.

Table 4. Rat liver ferritin radioactivity 2 and 72h after administration of [¹⁴C]leucine (5 μCi/100 g) in normal rats (group I) and in rats with acute blood loss (group II)

Values are expressed as mean c.p.m./liver±2 s.e.m. Ferritin radioactivity in group II was measured 2 days after blood loss, when the rate of loss of iron from ferritin is maximal. Twelve animals were used in each group.

	Normal rats (Group I)			Bled rats (Group II)		
	Period after [¹⁴ C]leucine administration		% loss in label	Period after [¹⁴ C]leucine administration		% loss in label
	2h	72h		2h	72h	
	Ferritin radioactivity (c.p.m./liver)	Ferritin radioactivity (c.p.m./liver)		Ferritin radioactivity (c.p.m./liver)	Ferritin radioactivity (c.p.m./liver)	
	6400±835	4080±629	36	4560±529	3010±501	34

The mechanism of the stimulation of ferritin synthesis by iron has been the subject of many studies, and the following have been postulated. (1) Iron causes an apparent rise in ferritin synthesis by stabilizing apoferritin or its precursors against breakdown by proteolysis. Although originally postulated by Granick (1946), several studies have supported this model. Thus apoferritin was shown to be more susceptible than ferritin to proteolysis by pepsin (Mazur & Shorr, 1950b). Also, the loss of radioactive label from liver ferritin was decreased when iron was administered continuously to rats over a period of 4 days (Drysdale & Munro, 1966). Finally, Pape, Multani, Stitt & Saltmann (1968) have suggested that the spherical iron micelle of ferritin serves as a stabilizing template for apoferritin synthesis. (2) Iron initiates apoferritin synthesis by activation or derepression of the regulatory gene, thus causing as a necessary preliminary the synthesis of new mRNA. This view of ferritin synthesis has been suggested by studies showing that actinomycin D, when administered to the intact animal, caused a decrease in [14 C]-leucine incorporation into ferritin *in vitro* and *in vivo* (Yoshino *et al.* 1968; Yu & Fineberg, 1965). (3) Iron causes an increase in the rate of apoferritin synthesis by affecting the rate of translation of mRNA. This type of control has been suggested by several authors, although detailed descriptions of the mechanism involved have differed (Drysdale *et al.* 1968; Chu & Fineberg, 1969).

The results of the present study are consistent only with the type of mechanism described by (3) above. The absence of a stabilizing effect by iron is demonstrated by the results of studies with cycloheximide, an inhibitor of ferritin synthesis. The decrease in hepatic ferritin radioactivity in rats after administration of cycloheximide was not changed when iron was also administered. Further evidence that iron does not stabilize the protein moiety of ferritin against breakdown may be inferred from the experiments in which ferritin turnover was measured after acute blood loss. The breakdown of ferritin, measured as the percentage decrease in total radioactivity between 2 and 72 h after injection of [14 C]leucine, was the same in bled rats as in normal rats (35%). Since the 2 h incorporation was significantly lower in the bled animals, the rate of synthesis was probably less in that group.

The present study has also shown that actinomycin D does not inhibit the increase in ferritin synthesis caused by iron. Results from experiments with actinomycin D must be interpreted with caution, since the doses used decrease total RNA synthesis by only 80% (Drysdale & Munro, 1966), and since the effect of the drug on different types of RNA may vary. However, we consider it

unlikely that iron acts by activation or derepression of the regulatory gene for apoferritin synthesis, or that new mRNA synthesis is a necessary preliminary to increased ferritin synthesis, as postulated by Yoshino *et al.* (1966).

Several comments may be made about the experimental evidence supporting the alternative mechanisms (1) and (2) above. Pepsin hydrolysis of apoferritin and ferritin has been reinvestigated (R. R. Crichton, personal communication) and the results, in contrast with those of Mazur & Shorr (1950b) show that there is no difference in the rates of hydrolysis. The observations by Drysdale & Munro (1966) are not easily reconciled with the results of the bleeding experiment in the present study, but the data of these authors is difficult to interpret because of the small number of animals (three) in each of their experimental groups. The arguments of Pape *et al.* (1968) have been fully answered elsewhere (Drysdale, Haggis & Harrison, 1968).

Evidence for gene activation by iron rests on inhibitory effects of actinomycin D on ferritin synthesis (Yoshino *et al.* 1966, 1968; Yu & Fineberg, 1965). The doses of actinomycin D used in the present study were 1.0 and 1.5 $\mu\text{g/g}$ body weight. Higher doses, such as that of 1800 $\mu\text{g/g}$ used by Yu & Fineberg (1965) should be regarded as excessive, since they cause non-specific decreases in protein synthesis (Drysdale & Munro, 1966). A similar criticism may apply to the doses used by Yoshino *et al.* (1968) (2–4 $\mu\text{g/g}$). On the other hand, Yoshino *et al.* (1966) observed actinomycin-caused inhibition of ferritin synthesis in liver slices at a dose of 1 $\mu\text{g/g}$.

Control of ferritin synthesis apparently resides at the level of translation, but the exact nature of the controlling mechanism remains obscure. An attractive hypothesis, already suggested by Chu & Fineberg (1969) as a possible mechanism in HeLa cells, is that the synthesis of apoferritin *in vivo* is normally decreased by the presence of a specific protein repressor, which acts in the cytoplasm and is characterized by a high rate of turnover relative to ferritin. This hypothesis is prompted by the observation that actinomycin D causes an apparent increase in [14 C]leucine incorporation into rat liver ferritin (Table 2). A similar synergistic effect has been noticed in several enzyme systems, both *in vivo* (Rosen, Raina, Milholland & Nichol, 1964; Della Corte & Stirpe, 1967) and *in vitro* (Papaconstantinou, Stewart & Koehn, 1966; Eagle & Robinson, 1964), and the phenomenon has been explained as selective inhibition of a cytoplasmic repressor by several authors (Garren, Howell, Tomkins & Crocco, 1964; McAuslan, 1963). Further, Ambrose (1969) has characterized a macromolecule that inhibited antibody response *in vitro*, and has suggested that it is indeed the postulated repressor. It is evident that

the most satisfactory test of the above model of ferritin synthesis would be isolation of the appropriate ferritin repressor.

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