MASTER

"IRON METABOLISM IN THE X-IRRADIATED ANIMAL"

Final Report

MASTER

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SUMMARY

The findings in our laboratory during the period of this research may be summarized as follows:

(1) Determination of changes in activity, per gm. of tissue, of the enzyme ferrochelatase is an accurate index of changes in erythropoietic activity of bone marrow and spleen in the rat.

(2) Changes in ferrochelatase activity in tissues of the spleen-shielded X-irradiated rat demonstrate the almost complete cessation of erythropoietic activity of bone marrow and spleen, the marked stimulation of spleen activity, which reaches values twice that in the normal rat, prior to falling to normal levels, and the gradual increase in bone marrow activity to normal pre-irradiation levels.

(3) Proof has been obtained for the biosynthesis of plasma transferrin on polysomes attached to the endoplasmic reticulum in the hepatic cell of the liver (the "rough E.R."). The experimental data also demonstrate that the nascent protein then passes on into the "smooth E.R." (vesicles which are free of polysomes) prior to secretion into the plasma. This mechanism helps to explain our finding that newly synthesized transferrin does not bind intracellular iron.

(4) The circulating red cell of the normal rat contains 6 multiple hemoglobins which have been separated by the method of isoelectric focusing gel electrophoresis. The relative distribution of these hemoglobins in bone marrow and spleen are the same, but differ from that seen in the adult red cell. Proof has been obtained which shows that these hemoglobins are not synthesized uniformly in marrow erythroid cells at all stages of maturation, i.e. hemoglobin V is associated mostly with the "youngest" erythroid cell whereas hemoglobins III and IV are associated mostly with the "oldest" cells.

1. Studies of the enzyme Ferrochelatase (Heme Synthetase).

Our interest in this enzyme, which catalyzes the incorporation of inorganic iron into protoporphyrin to form heme, was related to an attempt to find a diagnostic tool of biochemical significance with which to study the metabolism of iron in erythroid cells of the marrow, with special reference to the x-irradiated rat. This approach was meant to overcome our reliance on microscopic identification which is always associated with a certain amount of ambiguity and subjectivity. As we found, measurement of changes in ferrochelatase activity (per gm. of tissue) proved to be an accurate index of changes in erythropoietic activity.

Two different assay methods for this enzyme are available. One is a spectrophotometric method, rapid and comparatively easy to perform. The second is more cumbersome and time-consuming, involving the isolation of ^{59}Fe -labelled heme, using carrier heme, from an incubation mixture containing ^{59}Fe salts and protoporphyrin. The first method, although satisfactory when studying purified enzyme preparations can give ambiguous results when using crude tissue homogenates or tissue extracts. The second method yields more consistent results with crude tissue homogenates.

Attempts to purify ferrochelatase from rat reticulocytes revealed that the enzyme was associated with membrane lipid and required the use of detergents, e.g. deoxycholate, for solubilization and expression of enzyme activity. Chromatography on cellulose columns yielded fractions with a much enhanced specific activity of the enzyme but the overall recovery of enzyme from the crude extract was very low. The apparent loss of enzyme activity was due to the fact that much of the enzyme was separated on the column from phospholipid, thereby

eluting as inactive fractions. If such fractions were supplemented with phosphatidyl choline, in the form of a sonicated suspension in aqueous medium, activity of the enzyme was recovered. Other pure phosphatides studied were found to be inactive or much less active than phosphatidyl choline. Further attempts at purification of this enzyme were abandoned since they did not appear to be related to our immediate research.

II. Ferrochelatase activity as an index of erythropoetic activity.

Treatment of rats with phenylhydrazine, cobalt chloride, human urinary erythropoetin or by chronic blood loss resulted in a marked elevation in ferrochelatase activity per g. of spleen (3.2 - 5.6 x normal) . In all instances liver ferrochelatase activity remained unchanged, despite the presence of this enzyme in relatively high quantities in the mitochondrial membrane. On the other hand, rats bearing the Walker 256 Carcinosarcoma, in addition to sustaining an increase in enzyme activity in the spleen (1.7 x normal) showed an increased ferrochelatase activity in the liver (1.6 x normal) and a marked decrease in enzyme activity in the bone marrow (0.3 x normal). These latter results for tumor-bearing rats, taken together with previous findings of inhibition of marrow protein and nucleic acid synthesis, suggests that the marrow is a specific target of inhibition whereas the spleen and liver are stimulated to overcome the lack of heme synthesis in the marrow. Treatment of rats by the methods listed above (except for the tumor-bearing rats) resulted in marked stimulation of ferrochelatase activity in bone marrow cells, confirming the well known erythropoetic stimulation which occurs in those situations.

In rats treated with phenylhydrazine or by bleeding, it was demonstrated that increased ferrochelatase activity of the spleen was associated with an increased capacity to incorporate 2- ^{14}C -glycine into both heme and globin of hemoglobin in vitro. These results confirmed the conclusion that alterations in spleen ferrochelatase activity are due to alterations in erythropoietic activity and are not due to the activity of the spleen as a scavenger of dead red cells or as a sequestering agent for reticulocytes. Instead the rat spleen has an active erythropoietic function which appears to have a "back up" function when marrow activity is inhibited.

In all experiments listed above, with the notable exception of rats receiving cobalt chloride, the size and weight of the spleen was increased. Although this has frequently been ascribed to an engorgement of this organ with damaged red cells, in all instances, including rats treated with cobalt chloride, there was noted an increased rate of incorporation of ^{14}C -thymidine into total DNA and of ^{14}C -orotic acid into total RNA. The results obtained with rats treated with cobalt chloride emphasized the fact that an increased rate of DNA and RNA synthesis was taking place independent of tissue growth. That the stimulation of nucleic acid synthesis was real was confirmed by our finding that administration of cobalt chloride together with Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, resulted in abolition of the increased rate of RNA synthesis as well as of the increased ferrochelatase activity in treated rats.

Finally, we noted a marked stimulation in turnover of the basic histones in the spleen of rats treated with phenylhydrazine or cobalt chloride, as measured by the relative incorporation of ^{14}C -lysine into these proteins. These results confirm the metabolic relationship between these basic nuclear proteins and DNA and confirm our conclusion that the

stimulated spleen is undergoing an increase in multiplication of erythroid cells.

III. Ferrochelatase activity changes in tissues of x-irradiated rats.

Rats whose spleens are exteriorized and shielded with lead during whole body exposure to an otherwise lethal dose (900r) of x-irradiation recover from the initial damage to their bone marrow. Ferrochelatase activity of various tissues from such animals, from those not subjected to irradiation, as well as from rats exposed to irradiation in the absence of spleen lead-shielding were measured. The results may be summarized below:

(a) Marrow. Activity of the marrow after x-irradiation of spleen-shielded rats fell to very low levels (2 days) but gradually returned to normal values after some 30 days. This behaviour of the enzyme appears to mirror exactly the results that have been obtained by microscopic examination. Unshielded rats died within 7-8 days.

(b) Spleen. Activity of ferrochelatase in the spleen of shielded rats also declined to very low values soon after irradiation but began to increase after some 8 days reaching values twice that in normal rats on the 17th day and returning to normal values by the 30th day.

(c) Liver. Ferrochelatase activity of the liver of irradiated rats fell by 25% on the second day but soon rose to reach a maximum level that was somewhat higher than normal (by 25%) and returned to normal values by the 30th day.

(d) Circulating red cells. Enzyme activity in circulating red cells fell to low values on the 8th day, rose to values twice that of normal rats on the 17th day, returning to normal values by the 30th day.

These results strongly suggest that this enzyme assay method can be used to obtain accurate biochemical information with respect to damage to heme-synthesizing cells due to irradiation as well as the rate of recovery of such cells after irradiation. The "overshoot" of enzyme activity in spleen suggests that this organ is stimulated, in the absence of bone marrow erythroid activity, to proliferate its erythropoietic cells. A similar though less marked effect in the liver suggests that even in the adult rat, this organ may still contain some small residue of erythroid cells sensitive to irradiation or that irradiation damages the heme-synthesizing enzymes required for synthesis of the cytochromes and catalase. The initial loss of ferrochelatase activity in the circulating red cells of the irradiated rat is a mirror of the loss of erythropoietic activity of the marrow, whereas the subsequent "overshoot" of enzyme activity in these cells is a mirror of the ability of the spleen to "seed" the marrow as well as its own stimulated ability to produce erythroid cells.

That changes in ferrochelatase activity of the spleen are an index of altered metabolism of erythroid cells is further confirmed by comparing the ability of respiring spleen slices to incorporate $^{14}\text{-C}$ thymidine into total DNA with its ability to incorporate ^{59}Fe into hemoglobin and ferritin in irradiated rats. These patterns are all complementary on each of 4 test periods corresponding to days 2, 7, 14 and 21 following irradiation.

IV. Biosynthesis of multiple hemoglobins in the rat.

This study grew out of attempts to relate hemoglobin synthesis to the effects of x-irradiation on enzymes in the marrow erythroid cell. Small quantities of isotopically-labelled hemoglobin had to be isolated in a pure state and we therefore resorted to the technique of isoelectric focusing on acrylamide gels. This method yielded 6 well-defined hemoglobin

bands, with the largest quantity present only to the extent of 36%. Repeated analyses by this method of re-crystallized hemoglobin from circulating red cells of a large number of individual rats revealed the same 6 hemoglobins and in much the same relative quantities, regardless of age, sex and strain, including several inbred strains. Examination of the hemoglobin extracted from marrow and spleen revealed the same 6 hemoglobins. However, in marrow and spleen these hemoglobins had a different distribution, especially with reference to fractions III, IV, and V as shown in the following table:

<u>Hb</u>	<u>Isoelectric Point</u>	<u>Red Cells</u>	<u>Marrow</u>	<u>Spleen</u>
I	7.96	9%	9%	8%
II	7.75	15	12	13
III	7.58	21	7	13
IV	7.32	36	27	24
V	7.13	12	37	38
VI	7.09	7	7	4

All 6 hemoglobins are tetramers and of equal molecular weight. Quantitative amino acid analyses reveal that they are very similar. Current studies in our laboratory are aimed at identification of the differences in primary amino acid sequence among the chains of these multiple hemoglobins. Of further interest was the absence of a "fetal type" of hemoglobin. Up until the 14th day of pregnancy the hemoglobins in the circulation of the fetus are completely different from any of the adult hemoglobins, as measured by isoelectric focusing analysis. On day 14 hemoglobin II appears and by day 16 all 6 hemoglobins are present together with several of the early (embryonic?) hemoglobins. By day 17 the pattern and distribution of the 6 adult hemoglobins has been established and remains unchanged at birth and thereafter during the life of the rat.

The most interesting finding arose from an attempt to determine whether the extent of biosynthesis of the 6 hemoglobins at all stages of maturation of the marrow erythroid cell remained the same. In order to answer this question it was necessary to demonstrate that a suspension of mixed marrow cells of all ages would incorporate ^{59}Fe into the 6 hemoglobins to an extent proportional to the relative amounts of each present, i.e. that their specific radioactivities would be equal. This was demonstrated by in vitro incubation of total marrow with serum-bound ^{59}Fe and subsequent analysis of each of the 6 hemoglobins extracted from the total cells. For the in vivo experiments rats were injected intravenously with serum-bound ^{59}Fe and blood samples were removed from the tail vein at various times. Essentially all of the isotope was cleared from the plasma in 6 hours, the earliest sampling time. The samples withdrawn were small (0.2-0.3 ml.) in order to avoid stimulation of erythropoiesis. The 6 hemoglobins were separated and their specific radioactivities determined. At the early sampling times (0.5 - 1.0 days) the specific activities of fractions III and IV were equal and the highest, whereas that of fraction V was the lowest (one-third of fraction IV) and the others in-between. With time the specific activities of all hemoglobins reached the same value. These results are best interpreted as meaning that the biosynthesis of hemoglobin V (the major hemoglobin in a mixed population of marrow or spleen cells) was associated to the greatest extent with the youngest of the erythroid cells whereas that of hemoglobins III and IV was associated with the oldest of the erythroid cells. The results also indicate that there is no complete cessation of biosynthesis of hemoglobin V even in the oldest erythroid cells since reticulocytes incorporate ^{59}Fe into hemoglobin V.

These results, demonstrating a non-uniform biosynthesis of multiple hemoglobins in the rat can be explained by either of two mechanisms. First, it is possible that the erythroid cells of all ages contain the genetic information to make all chains required for the 6 hemoglobins, but are expressed and repressed at different stages of maturation of the erythroid cell. The other possibility assumes that enzymes appear (or disappear) during cell maturation which can act on the chains resulting in some minor changes in amino acid composition, e.g. conversion of a carboxylic acid group of glutamic and/or aspartic acid to the corresponding amides, or vice versa. An understanding of the differences in their primary structure may help to choose between these alternatives.

V. Biosynthesis of Transferrin, the plasma iron-binding protein.

Transferrin was purified from pooled rat serum after saturation with ^{59}Fe . After a preliminary separation on Sephadex the material was subjected to preparative isoelectric focusing in a column containing both pH and sucrose density gradients. Two salmon-pink bands, which accounted for all of the radioactivity, were removed and subjected to analysis. Their isoelectric points were different and the major transferrin was present to the extent of two-thirds of the total. Each protein consisted of single polypeptide chains with identical molecular weights and their amino acid composition was very similar. Furthermore they were immunochemically identical as tested by the method of immunoelectrophoresis against a rabbit antiserum directed against one of these. It was concluded that the minor component probably represented a genetically different species. Both transferrins bound 2 atoms of iron per mole.

Biosynthesis of transferrin was followed by a short period of incubation of normal rat liver slices with an amino acid mixture in which the leucine was labelled with tritium. The washed slices were now re-incubated with a complete amino acid mixture containing unlabelled leucine. The homogenized liver samples were treated to separate free (unattached) ribosomes from attached ribosomes (attached to the endoplasmic reticulum; "rough" E.R.) as well as from "smooth" E.R. (free of ribosomes). The transferrin was precipitated from each fraction by use of the specific rabbit antiserum after removal of any radioactivity which was precipitated by the addition of antiserum to whole chicken serum and chicken serum. The latter precipitate contains small amounts of radioactivity which is adsorbed to the antibody-antigen precipitate and lowers or eliminates any non-specific radioactivity which might be adsorbed when antiserum to transferrin is added to the sample. Pure transferrin was added as carrier.

The results may be summarized in the following:

(a) The results clearly indicate that transferrin, like other serum proteins reported in other laboratories, is assembled on the rough endoplasmic reticulum and not on the free polysomes.

(b) At each time interval when samples were analyzed the smooth E.R. also contained nascent transferrin but yielded a time-curve of incorporation below that of the rough E.R. However after 50 minutes the values for smooth E.R. were higher than those for rough E.R. These results are consistent with the interpretation given by other workers for other serum proteins, that transferrin is assembled on polysomes attached to E.R., pass into the lumen of these vesicles and appear in the smooth E.R. prior to their movement out of the liver cell into the circulating plasma.

(c) After 30 minutes of incubation labelled transferrin appeared in the incubation medium in increasing amounts suggesting that this represented the secretion of the newly synthesized protein into the plasma.

The foregoing experiments were carried out with the aim of explaining how transferrin could be synthesized inside the liver cell and yet escape binding hepatic intracellular iron, given the very great avidity of transferrin for iron. Identical "chase" experiments were now performed with the exception that serum-bound ^{59}Fe was added to the initial incubation mixture instead of radioactive leucine. Examination of the transferrin isolated by means of its specific antibody and carrier transferrin, revealed that there was essentially no ^{59}Fe on the newly synthesized transferrin. Because the levels of ^{59}Fe in these precipitates were so low as compared with the ^{59}Fe in the total sample a second aliquot was treated with antiserum to chicken serum and chicken serum and the washed precipitate counted in order to determine whether the counts seen in the transferrin antibody-antigen precipitate were significant. Both aliquots gave identical values within limits of error of the technique.

These results, taken together with the data obtained in the studies of transferrin biosynthesis, strongly suggest that the reason why nascent transferrin does not bind intracellular iron is due to the fact that the protein molecule, as it is elongated, is inserted inside the lumen of the rough E.R. thereby being unavailable for iron-binding. With time, the transferrin molecule passes along the E.R. until it finds itself in that portion of the E.R. which does not have attached polysomes ("smooth" E.R.).

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