STERCOBILIN N^{15} EXCRETION IN REFRACTORY ANEMIA

By G. WATSON JAMES, III, M.D., and (*by invitation*) LYNN D. ABBOTT, Jr., Ph.D.

RICHMOND

In his Harvey Lecture delivered October 21, 1948, Cecil J. Watson concluded with a statement which said in part, ". . . one may anticipate that the use of isotopes in the study of urobilin and stercobilin will shortly permit solution of many hitherto perplexing questions."[1] This prediction was to prove correct, for both heavy nitrogen and radioactive carbon have been used by many investigators in both this country and abroad to study the problems associated with the urobilinoid pigments. Great progress has been made in the study of erythrocyte life span, porphyrin metabolism and many other facets of pigment metabolism through the application of fundamental biochemical knowledge, in itself, revealed by isotopic research. Presentation of some data on the excretion of stercobilin N^{15} in refractory anemia might aid in the study of fecal stercobilin.

Stercobilin has at least two metabolic sources, the first, well known, comes from the catabolism of ferro-protoporphyrin (heme) of the circulating erythrocytes. It is 80–85% of the total fecal stercobilin. A second source, not as well known, or thoroughly understood, seems in part to be associated with erythropoiesis, but perhaps not entirely. It is this early anabolic fraction that has our interest for the moment. It is 15–20% of the total fecal stercobilin. This source was not at all suspected until London, *et al.* demonstrated its presence in an N^{15}-isotopic study aimed at following the degradation of N^{15}-labeled erythrocytes.[2] Studies by many investigators have confirmed amply this finding.[3, 4]

In essence, the clinical investigation involves the oral feeding of labeled N^{15}-glycine to tag the nitrogen of the pyrrole ring. Following a pathway demonstrated by Shemin and his fellow workers,[5] the nitrogen enters a succinate-glycine cycle, condenses with 'active' succinate to form *alpha*-amino, *beta*-ketoadipic acid, and a decarboxylation occurs to result in *delta*-aminolevulinic acid. Two moles of the latter combine under the influence of a specific dehydrase to form a monopyrrole porphobilinogen. This last step, the formation of a heterocyclic ring structure from an aliphatic chain is a marvelous bit of biochemistry, inherent in all living tissue. Porpho-

From the Laboratory for Clinical Investigation, Department of Medicine and Department of Biochemistry, Medical College of Virginia, Richmond

Aided in part by Grant A-1139, U. S. P. H. Service

bilinogen, through condensation with itself, gives rise to the cyclic tetrapyrroles, and the labeled nitrogen finally resides in the pigment portion of the hemoglobin molecule. In this manner the erythrocyte is labeled and its life span studied.

In a typical study in our laboratory, a young medical student was fed 24 grams of N^{15}-labeled glycine (31 atom % excess N^{15}) over a 48-hour period. Samples of venous blood were taken at spaced intervals for isolation of hemin. All his stools were collected in four-day metabolic periods for five to six months for isolation and crystallization of stercobilin. The N^{15} content of the hemin and stercobilin was determined in the mass spectrometer. In such a study, one finds the heme tag of the red cells rising for about twenty days. It reaches a plateau for about seventy days and then declines for the next forty to sixty days. As the labeled erythrocytes disappear from the circulation, the N^{15} label on the fecal stercobilin begins to rise, reaches a peak about the 115th to 140th day and then declines. This rise and fall is a consequence of the destruction of the labeled erythrocytes. It thoroughly demonstrates a catabolic source of a major part of the fecal stercobilin.

Study of the entire stercobilin labeling curve, however, shows additional findings. In the initial four-day period and sometimes in the second four-day period, stercobilin bears a label higher than that of the circulating heme. *Circulating* red cells cannot be the source of this "early" fraction of labeled fecal stercobilin, unless there is a very short-lived population, as claimed by Evans.[6]

The possible sources of the early labeled stercobilin, sometimes called 'anabolic' stercobilin seemed at first to be most likely related to erythropoiesis. It might arise from destruction of immature or newly formed red cells before they have entered the circulation. This would be much like an "infant mortality." Experimental evidence has also suggested that porphyrin synthesis and globin synthesis are disassociated in the developing erythroblast and thus these cells might be synthesizing and degrading protoporphyrin simultaneously. The destroyed protoporphyrin or heme is converted into biliverdin-bilirubin and then by the bacterial flora of the gastrointestinal tract into one of the urobilinoid pigments, principally stercobilin. It is recognized at the present time that bilirubin is an obligatory intermediate in stercobilin formation. However, not all of the heme pigments are in the red cell. There is myoglobin and there are other heme-containing proteins of intracellular enzyme systems, such as catalase, peroxidase, and the cytochromes. London has considered that the turnover rate of these sources is too slow to account for such a rapid appearance of the fecal stercobilin label.[2] If a requirement is a rapid cellular metabolism and turnover and this would certainly seem to be a logical deduction, then one might suspect the liver, gastrointestinal epithelium, lymph node or leuko-

cyte formation could be in part responsible for some of the early fraction of stercobilin. It would be necessary to show that these cells were synthesizing porphobilinogen, since this has to be considered also as an obligatory intermediate in bilirubin synthesis.

Study of patients with diseases of the blood forming tissues should be an approach to the anabolic stercobilin problem. Relabeling that could occur when large quantities of N^{15}-glycine was fed, was a difficulty. The glycine pool of the body, with a high label lasting for several days, could relabel over a longer period of time and easily confuse interpretation of experimental results. We avoided this in part by using only one gram of N^{15}-glycine for oral feeding. In patients with active erythropoiesis, we have labeled the red cells and stercobilin with as little as one gram of either 31 or 60 atom percent excess N^{15}-glycine.[6, 7] That we could label with as little as one gram of N^{15}-glycine in a normal individual, was demonstrated in our own red cells. We found the life span could be measured. A medical student volunteered to collect his stools for a stercobilin labeling experiment, and a subsequent study in a normal young woman was performed also. When one gram of N^{15}-glycine was fed to normal subjects, we observed similar findings as were found when the larger 24-gram quantity was used. The stercobilin label increased with the disappearance of the tag from the circulation and what was more important for us—an increased label could be detected on the early fraction of stercobilin. This meant it was feasible to study patients with various hematologic disorders without having the burden of a large pool of labeled glycine. The concentrations in the hemin and the stercobilin were small, but they were measurable.

Patients with refractory anemia, that is, those who persisted in a transfusion requirement in spite of all present available therapy, were given orally one gram of labeled glycine in a manner similar to the normal subjects. Dobriner and Rhoads found that patients with refractory anemia excreted increased quantities of both coproporphyrin and urobilinogen in the stool.[9] In addition, according to Davidson, refractory anemia patients could be classified according to the cellularity of the bone marrow.[10] If we were to undertake a study of refractory anemia patients with erythroid *hyper*plasia and erythroid *hypo*plasia, some information might be elicited.

In general, those patients with refractory anemia, who have a hyperplastic erythroid marrow, frequently have an accompanying myeloid and megakaryocytic hyperplasia. In some the erythropoiesis is megaloblastoid. This proved to be true in three of the patients of this type whom we studied. In light of the subsequent course of two of these patients, the myeloid hyperplasia, with giant metamyelocytes and occasional myeloblasts is perhaps of some significance. Of the three patients with *hypoplasia* of the marrow, one appeared to have erythroid *aplasia*. The second was a child with idiopathic

aplastic anemia. The other was a young man with erythroid hypoplasia; although there was some normoblastic erythropoiesis, he had an accompanying myeloid and megakayrocytic hypoplasia.

Representative of the patients with erythroid *hyperplasia* was E. W. S., 59, a retired naval officer. He first experienced symptoms of anemia three years before he came to our attention. Having been studied in several clinics on the East Coast, various diagnoses, ranging from early Hodgkin's disease to chronic di Guglielmo's disease, had been offered. There was a bi-monthly or fortnightly transfusion requirement of 500 ml. of blood. Hemosiderosis, either as a part of this syndrome or iatrogenic from transfusion, was beginning to be apparent. The results are shown in the graph in Fig. I. This figure contrasts the findings in the normal male, showing open columns for stercobilin N^{15} concentration in atom percent excess and the solid line for the uptake in the circulating heme. There was a marked difference. The patient, E. W. S., demonstrated a large label in the first four-day metabolic period and an even higher tag in the second metabolic period. A rapid decline occurred for the next three periods. In contrast, the normal man had a much smaller label in the first period, a decline in the second. The last three periods were nearly constant. This indicated to us that the stable state had been reached, and the label entering stercobilin during this time was probably coming from an equilibrated isotopic source.

Clearly, the enhanced label in the early fecal stercobilin of the patient could not be coming from the circulating erythrocytes, even though his newly formed red cells did have a greater tag than that found in the normal

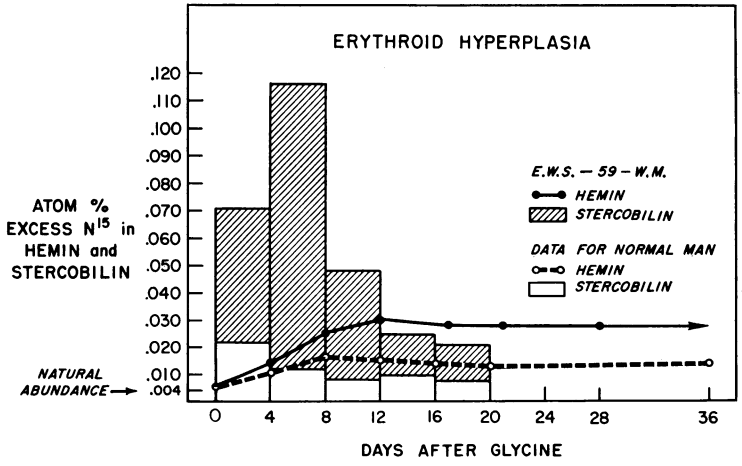

FIG. I: N^{15} stercobilin and N^{15} hemin in patient E. W. S. with hyperplastic bone marrow.

TABLE I

Hematologic and Pigment Metabolism Data

	D. S. 24 W. M. Normal	E. W. S. 59 W. M. Erythroid Hyperplasia	W. S. 71 W. M. Erythroid Aplasia
Hemoglobin gms./100 ml	15.7	10.2	7.6
Red Cell Count cu. mm	5,490,000	3,290,000	2,260,000
Hematocrit %	48	30	19
White Cell Count cu. mm	7,400	5,850	2,400
Platelet Count cu. mm	normal	166,000	75,000
Reticulocytes %	1.5	1.4	0.2
Serum Bilirubin mg./100 ml			
Free	—	0.1	0.1
Conjugated	—	0.5	1.2
Fecal Urobilinogen mg./day	107	318	175

man. Nevertheless, this red cell label is not as great as it seems, since the patient's total circulating hemoglobin was lower, and consequently the total circulating heme N^{15} was smaller. Similarly the label in the stercobilin is larger because his daily fecal stercobilin was roughly twice that of the normal man. (Table I)

In neither the normal nor the anemic man was there any evidence of an immediate or random destruction of the circulating red cells. The average life span in the normal man was about 120 days and that for the patient about 80 days.

The bone marrow of E. W. S. showed an extremely primitive type of erythropoiesis. Giant normoblasts were present; there were frequent reticulum cells, some megaloblasts, and an accompanying myeloid hyperplasia with giant metamyelocytes. Megakaryocytes were frequent. Table I gives more complete hematologic data, as well as changes found in serum bilirubin and fecal urobilinogen.

Because of the findings in the bone marrow, the source of the early labeled "anabolic" stercobilin did not exclude marrow destruction of primitive red cells. These results suggested that the early fraction of stercobilin might well be associated with erythropoiesis. Similar study in four additional patients gave almost identical findings.

An opportunity soon presented itself to study a patient with a refractory anemia who had a *hypocellular* marrow. He was a 71-year old retired lithographer who had been suffering from anemia and weakness for seven years before the study was performed. The original diagnosis had been systemic Hodgkin's disease. Fortnightly, transfusions kept him comfortable and free from angina which was one of his chief complaints. He agreed to the study. The peripheral blood findings and other data are in Table I. Insofar as we

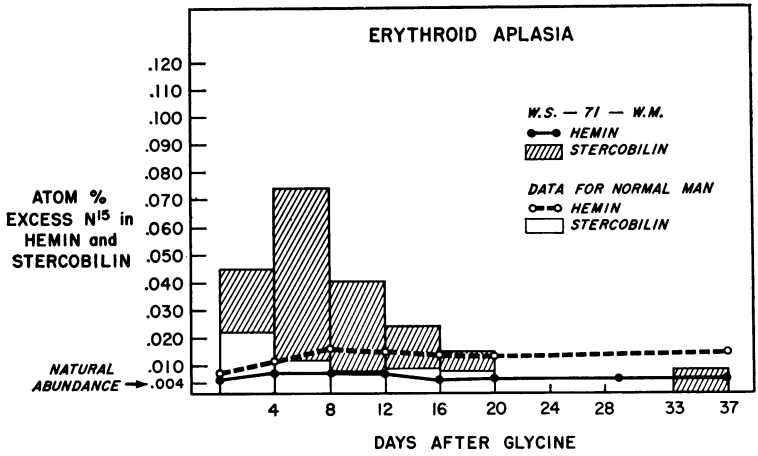

FIG. II: N^{15} stercobilin and N^{15} hemin in a patient W. S. with erythroid aplasia.

could determine, there was an almost complete erythroid *aplasia*. Sternal marrow was virtually devoid of any cells that resembled erythrocyte precursors, but a few reticulum cells and white cell precursors were present. The results are shown in Figure II, and the graph is constructed in an identical manner as Figure I. The stercobilin label in the normal is in open columns and the fecal stercobilin in the patient is represented by hatched columns. As in the patient with the hyperplastic marrow, this subject, in whom there was virtual erythroid *aplasia*, demonstrated enhanced N^{15} incorporation in the first metabolic period which increased during the second metabolic period and then gradually declined. Of special importance was the uptake of N^{15} in the circulating red cells. Virtually no label occurred. This finding was in keeping with the reticulocytopenia and the appearance of the bone marrow. Obviously, no evidence of peripheral destruction could be obtained.

The two additional patients with hypocellular marrows showed similar findings with the exception that in both of the others there was a definite uptake in the circulating red cells in spite of the finding of reticulocytopenia. The hypocellular marrow showed erythroid *hypoplasia* rather than erythroid aplasia.

These comparative investigations suggested to us that the early fraction of stercobilin, showing an enhanced label in both the patient with the erythroid *hyperplasia* and the erythroid *hypoplasia*, was quite likely not *totally* related to active or even primitive erythropoiesis.

The rapid appearance of the label in stercobilin in the first metabolic period, which must represent labeling that occurred in the first twenty-four or forty-eight hours, indicates the formation of tetrapyrrole by a very rapid

and active metabolic process. It is not possible from the available data to name an individual tissue responsible for these findings. Earlier it was suggested that liver, gastrointestinal epithelium, lymphatic or white cell precursors, might be implicated. The eventual fate of these two patients might perhaps shed some additional information of value. Both died with an acute granulocytic leukemia—E. W. S. six years after the onset of his illness, and W. S. about nine years after the first symptoms.

Several similarities existed both clinically and morphologically. Both were originally diagnosed as a "reticulum cell disease". In the bone marrow, both had reticulum cells, and young white cells, though the numbers varied greatly. Young leukocytes with an increased amount of cytoplasm have been shown by Cullity and Vannotti to be capable of forming large quantities of porphobilinogen.[11]

A tentative hypothesis may be outlined based on these studies, as well as available evidence from the work of Gilbertsen, *et al.*, with labeled fecal mesobilifuscin.[12] As seen in Figure III, the normal pathway for heme formation and subsequent bilirubin formation is through a series of cyclic tetrapyrroles. The exact mechanism whereby porphobilinogen is polymerized into a Type III uroporphyrinogen is not known, but under normal circumstances the direction is toward the production of a Type III isomer rather than a Type I isomer. Decarboxylation of the acetic and propionic acid side chains of the porphyrin ring eventually leads to a porphyrin with

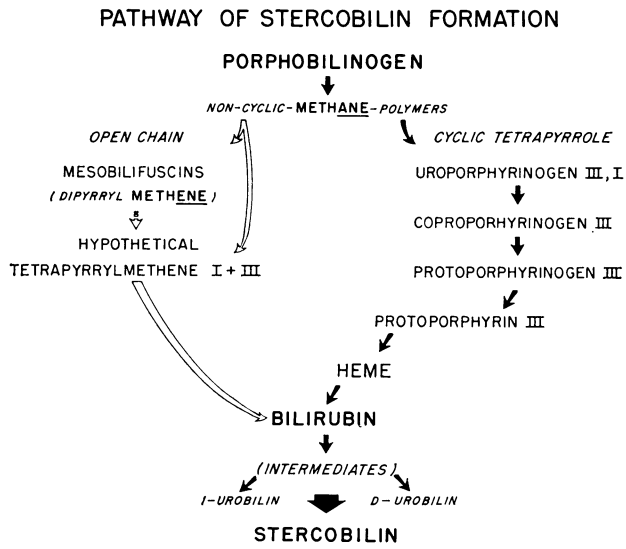

FIG. III: Normal pathway (black arrows) and hypothetical pathway (open arrows) from porphobilinogen to stercobilin.

methene linkages and four methyl, two vinyl, and two propionic acid side chains. This protoporphyrin chelates with iron to form heme and then hemoglobin. During this process, it is possible that the protoporphyrin formed might be degraded directly to bilirubin, excreted in the bile and the bilirubin transformed into stercobilin. Thus, a rapid or early labeled fecal stercobilin could be formed in a process directly related to erythropoiesis. There is no evidence at present that either uroporphyrinogen or coproporphyrinogen can be converted directly to bilirubin. One of the difficulties comes in an early step, for if porphobilinogen were to polymerize stepwise to form a tetrapyrrole and then close the ring, it would lead to a Type I uroporphyrinogen. This porphyrin, excreted primarily in the urine, does not appear in any significant quantities in a normal erythropoiesis. It does appear in situations in which there is either an abnormally stimulated red cell formation or in a genetic disturbance of porphyrin metabolism. Likewise, as yet no heme has been demonstrated that is not of the Type III protoporphyrin configuration.

Consider, on the other hand, a patient in whom the marrow is devoid of active erythropoiesis and in whom there is evidence of increased fecal stercobilin excretion. At the same time, isotopic study shows the increased label on the "early" stercobilin. How might this be explained? Gilbertsen, et al., have demonstrated that dipyrrylmethenes, the mesobilifuscins, are labeled early in a manner similar to the early "anabolic" fecal stercobilin. The label on a still unpurified mesobilifuscin fraction occurs earlier than the label on the fecal stercobilin. Although it cannot be proven that these mesobilifuscins are not catabolic from tetrapyrrylmethenes by splitting, the evidence recently presented, is against this idea.[12] Hypothetically, one might suppose that an active metabolic tissue, say leukocyte precursors or even early undifferentiated reticulum cells (in general not easily aspirated from marrow), which are able to synthesize porphobilinogen, also might be capable of polymerizing porphobilinogen to a dipyrrylmethene or tetrapyrrylmethene. The side chains might become reduced to give rise to a bilirubin, which is subsequently excreted in the bile and reduced by the gastrointestinal bacteria to a stercobilin. Stercobilin crystallizes with ease and in our present knowledge is a product of the opening of a Type III ring structure at the α-methene bridge. If a step-wise tetrapyrrylmethene is formed, it should correspond to a "type I" structure. This then would lead after side chain reductions to a "type I" stercobilin.

Insofar as our chemical studies have been carried out, the stercobilin isolated from the patients with the *hypoplastic* marrow was like stercobilin isolated from the patients with the *hyperplastic marrow*. Some patients were or had been on antimicrobials at the time of the study, and in some instances, the urobilinoid pigment was *d*-urobilin or a mixture of *d*-urobilin and ster-

cobilin; nevertheless, we have, as yet, no evidence that the stercobilin was different. It is an important point to be investigated.

These studies may be summarized as follows:

Patients with refractory anemia and erythroid hyperplasia excrete an increased fraction of early or "anabolic" fecal stercobilin. Likewise, patients with refractory anemia and erythroid hypoplasia or aplasia show an increased fraction of "anabolic" stercobilin. Thus, the anabolic stercobilin may be in part from a *non-erythroid* source. This source is from a tissue which must be actively synthesizing porphobilinogen in large amounts. An hypothesis for an alternate pathway from porphobilinogen through dipyrrylmethene polymers and thence to bilirubin and eventually to stercobilin is discussed.

Acknowledgements. We are very grateful to Dr. William T. Ham, Professor of Biophysics, and his staff, Mr. Ray Williams, Mr. Harry Mueller and Mr. Fred Schmidt, for their help in the N^{15} analysis in the mass spectrometer.

REFERENCES

1. WATSON, CECIL JAMES: Urobilin and stercobilin, The Harvey Lectures. Series XLIV, 1948–1949. Springfield, 1950. Charles C Thomas.
2. LONDON, I. M., WEST, R., SHEMIN, D., AND RITTENBERG, D.: On the origin of bile pigment in normal man, *J. Biol. Chem., 184:* 351, 1950.
3. JAMES, G. W., III: Stercobilin and hematopoiesis, *Am. J. Clin. Nutrition, 3:* 64, 1955.
4. GRAY, C. H., NEUBERGER, A., AND SNEATH, P. H. A.: Studies in congenital porphyria, 2. Incorporation of N^{15} in the stercobilin in the normal and in the porphyric, *Biochem. J., 47:* 87, 1950.
5. SHEMIN, D.: The succinate-glycine cycle: the role of delta-amino levulinic acid in porphyrin synthesis. Porphyrin Biosynthesis and Metabolism, Ciba Foundation, London, 1953. Churchill.
6. EVANS, R. L.: Short lived red cells in normal individuals, *Nature, 173:* 129, 1954.
7. JAMES, G. W., III, AND ABBOTT, L. D., JR.: Erythrocyte destruction in sickle-cell anemia: Simultaneous N^{15}-hemin and N^{15}-stercobilin studies, *Proc. Soc. Exper. Biol. & Med., 88:* 398, 1955.
8. JAMES, G. W., III, MOON, J. H., AND ABBOTT, L. D., JR.: Unpublished results.
9. DOBRINER, K., RHOADS, C. P., AND HUMMEL, L. E.: The excretion of porphyrin in refractory and aplastic anemia, *J. Clin. Invest., 17:* 125, 1938.
10. DAVIDSON, L. S. P., DAVIS, L. J., AND INNES, J.: Studies in refractory anemia, *Edinburgh Med. J., 50:* 226, 431, 1943.
11. CULLITY, B., AND VANNOTTI, A.: Porphyrin biosynthesis in the leucocyte, *Nature, 185:* 187, 1960.
12. GILBERTSEN, A. S., LOWRY, P. T., HOWKINSON, V. AND WATSON, C. J.: Studies of the dipyrrylmethene ('fuscin') pigments. I. The anabolic significance of the fecal mesobilifuscin, *J. Clin. Invest., 38:* 1166, 1959.

DISCUSSION

DR. CECIL J. WATSON (Minneapolis): It has been a real privilege for me to hear this fine report of Dr. James and his co-author, Dr. Abbott. I must say that I think

they are to be congratulated, for this is an extremely difficult basic type of study, in which there can be many pitfalls. I think they have carried it out in beautiful fashion.

One of the things that I would like to say about Dr. James' work—it doesn't relate only to this present study—is his great contribution in making available to us, or showing us how to use, N^{15} glycine in a much less expensive fashion. In our earlier studies at least we used rather large amounts and it is very expensive. He has shown that you can get by with as little as a gram of labeled glycine and get very respectable data, as he indicated this morning.

I would like to make two or three more comments and I can make them in much more expeditious fashion if I can show a couple of lantern slides—which I don't "just happen to have." [Laughter] It will just take me a moment.

May I have the first of those two slides, please.

[Slide] I just want to refer to the studies that Dr. James also mentioned of Dr. Sigrid Gilbertsen. She carried this study out on herself and this shows the early labeling of the dipyrrylmethene peak, in this instance just in advance of the early stercobilin labeling, and the lack of any significant labeling at the time of destruction of mature circulating red cells at 120 days in contrast to the very wide labeling of the stercobilin. With this finding in mind Dr. Gilbertsen studied patients with aplastic anemia and erythroid hypoplasia—

May I have the next slide, please.

[Slide] . . . and was able to show that the fecal mesobilifuscin, or dipyrrylmethene moiety frequently diminishes as the disease progresses. You see in this instance it went down to zero in spite of the fact that the fecal urobilinogen remained essentially the same or even increased slightly so that the ratio of urobilinogen to mesobilifuscin increased greatly and then finally to infinity.

I think it is safe to say that mesobilifuscin, or this type of dipyrrylmethene is mainly anabolic in character rather than catabolic. I would like to ask Dr. James if he feels that there really is any advantage, perhaps whether there isn't some disadvantage, to the concept of a tetrapyrrylmethene, or bile pigment being formed in this way to explain the early peak in the stercobilin rather than excessive formation of heme, perhaps in the developing red cells, the bone marrow, with destruction of heme and formation of bile pigment liberation from the young red cells. (This of course implies that there were islands, or foci of normoblasts in the present cases, such as have been demonstrated in similar instances.) We have been very much impressed with this possibility because of the clear observation that free porphyrins, can be released from developing red cells in the bone marrow. We haven't yet any proof that bile pigment is formed in normoblasts but I agree that it is entirely possible and I hope you will comment on what you think is the advantage between these two possibilities.

It would seem to me that if a bilirubin is to be formed independently of heme, in other words as a purely anabolic event, this would probably require a different enzyme system. One would anticipate that there would be some special enzyme for this kind of conversion to bilirubin, one that we, at the moment at least, have no use for in our internal economy, at least as far as hemoglobin metabolism is concerned. I think it is easy to see how, if there were an excess of porphobilinogen, there might well be formed small excesses of mesobilifuscin or dipyrrylmethene as an internal chemical artifact, if you will, without there being any necessity for an enzyme, but if you want to carry this farther to formation of bilirubin I doubt that this would be very likely unless there were another enzyme and I hope you will comment on this possibility. Thank you very much.

DR. RICHARD VILTER (Cincinnati): I would like to ask Dr. James whether I understood correctly that he thought this anabolic phase was going on in white cells and other cells in the bone marrow—there were no red cells—or is it going on in the liver and other parts of the reticuloendothelial system, for instance?

DR. JAMES (Closing): I would like to say that Dr. Watson has given me much help and encouragement through the years in these studies and I wish to tell him again how much I appreciate his aid. None of these investigations would have been possible had not Cecil James Watson in 1931 crystallized stercobilin for the first time. He was working in Hans Fischer's laboratory in Munich, and I think I know the excitement and thrill that he must have experienced in seeing these beautiful cadmium orange yellow crystals come floating down from the odorous brown liquors.

In the first patient, with hyperplastic marrow, I cannot say that heme is not the source, or a heme is not the source of the early anabolic stercobilin. We must assume that in the developing erythroblast, simultaneous degradation and synthesis may be occurring and that bilirubin (stercobilin) is arising from the heme in these developing red cells.

The point—and this partially answers Dr. Vilter's question, too—the point is that in the patient with erythroid aplasia we didn't find any developing red cells. Now, the two additional patients with erythroid aplasia showed almost identical changes. Granted, the one child with aplastic anemia did have on marrow concentration a few developing erythroblasts and the other patient, an idiopathic aplastic anemia, also had a few erythroblasts. The amount of erythropoietic tissue involved didn't seem to be sufficient to produce the magnitude of N-15 incorporation observed in the fecal urobilinogen excretion. That is why we have suggested that there may be an additional pathway where an appropriate bilirubin may be formed. If porphobilinogen polymerizes stepwise and closes to a ring, a uroporphyrin Type I results. If this were converted to a protoporphyrin type I then degraded to bilirubin, a different type stercobilin would result.

If our hypothesis is correct then there should be a stercobilin which is different from the stercobilin which arises as a destruction of bilirubin. There is no information along this line whatsoever and the studies that we have done so far indicate that the stercobilin formed from the patients with erythroid aplasia was just like the stercobilin formed from the patients with erythroid hyperplasia. We have not answered any questions. We have raised more.