STUDIES IN EARLY-LABELLED BILIRUBIN AND TISSUE HAEMS

A Thesis

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by

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PREFACE

The research comprising this thesis was carried out in the laboratory of Professor Lyonel G. Israels, in the Department of Internal Medicine, Faculty of Medicine, University of Manitoba at the Manitoba Cancer Treatment and Research Foundation. The inspiration, guidance, and friendship so generously provided by Dr. Israels are acknowledged with deep appreciation.

The collaboration of Drs. William Novak and Brent Schacter in several aspects of this research was a pleasant and stimulating experience. The expert technical assistance of Mrs. Diane Ollmann and Miss Mary Jack was invaluable in the preparation of these data. Special thanks are due Miss Nan Christie for her meticulousness and equanimity in the typing of this manuscript.

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CHAPTER I

INTRODUCTION

Bilirubin is known to originate largely as a catabolic product from the haem of senescent erythrocytes (Shribhishaj, Hawkins and Whipple, 1931; Hawkins, Shribhishaj, Robscheit-Robbins and Whipple, 1931; Hawkins and Johnson, 1939; James, Abbot, Norberg, Birkeland and Evans, 1953). Until less than twenty years ago there was no evidence for the existence of other sources of bile pigment, although bilirubin formation from haem precursors had been suggested by Whipple (1921). Bilirubin overproduction was, therefore, thought to occur only in the presence of an accelerated destruction of circulating erythrocytes.

In 1950 London, West, Shemin and Rittenberg and Gray, Neuberger and Sneath reported the isolation of labelled stercobilin from feces within five to twenty days following the oral administration of N^{15}-glycine to humans. This considerably preceded the loss of label from the haem of circulating erythrocytes. The labelled pigment could, therefore, not have arisen from the degradation of circulating haem. It was found to constitute eleven to twenty percent of the total stercobilin and has been variously designated as "Shunt" or "Early-labelled" pigment.

Clinical interest in early-labelled pigment was stimulated when it was shown to represent at least forty percent of the bile pigment excreted in one case of pernicious anaemia (London and West, 1950) and thirty to eighty-five percent in cases of congenital porphyria (Gray, Neuberger and Sneath, 1950 and London, West, Shemin and Rittenberg, 1950a). A similar phenomenon was documented in thalassemia (Grinstein,

Bannerman, Vavra and Moore, 1960). Subsequently, cases of hyperbilirubinemia of the overproduction type in the absence of a consistent degree of shortening of the circulating erythrocyte life span have been attributed to overproduction of early-labelled bilirubin (Israels, Suderman and Ritzmann, 1959 and Israels and Zipursky, 1962; Arias, 1962; Robinson, Vanier, Desforges and Schmid, 1962; Berendsohn, Lowman, Sundberg and Watson, 1964).

Further delineation of the mechanisms giving rise to this bilirubin required the development of more precise techniques. It was well known (Watson, 1956) that fecal stercobilin excretion was not an accurate, quantitative reflection of haemoglobin catabolism and underwent variations quite independent of changes in haem catabolism. In addition, bowel transit time of the pigment and difficulties inherent in quantitative collection introduced factors of delay and variability which obscured the temporal and quantitative kinetics of the appearance of early-labelled pigment. Methods were required to circumvent these difficulties.

Bilirubin, the direct metabolite of haem, replaced stercobilin in isotope determinations as methods were developed for its extraction from bile (Ostrow, Hammaker and Schmid, 1961) and plasma (Yamamoto, Skanderbeg, Zipursky and Israels, 1965). This was easily adapted to animal work through the use of total bile fistula preparations (Israels, Skanderbeg, Guyda, Zingg and Zipursky, 1963) and the use of human patients with biliary drainage tubes following common duct surgery. Intravenous "pulse" injection of the precursor, labelled with C^{14}, replaced oral administration

using N^{15}.

Application of these methods by Israels, Skanderbeg, Guyda, Zingg and Zipursky (1963) in dogs produced several findings: labelled bilirubin appeared in the bile within four to eight hours after the intravenous administration of 2-C^{14}-glycine and attained maximum specific activity within twenty-four to forty-eight hours. Ostrow, Hammaker and Schmid (1961) found the peak excretion of radio-labelled bile pigment in rats to occur between six and eighteen hours following injection of labelled glycine. The appearance of labelled pigment followed a similar pattern in man (Israels, Skanderbeg, Guyda, Zingg and Zipursky, 1963); labelled bilirubin appeared in both dogs and man before isotope was present in the haem of circulating red cells or marrow buffy coat.

Subsequently, Yamamoto, Skanderbeg, Zipursky and Israels (1965) reported the finding of a second peak of early-labelled bilirubin in the plasma of two normal human subjects following intravenous administration of 2-C^{14}-glycine. The first component (component I) was detectable at ninety minutes and the activity increased to reach a peak at twelve to twenty-four hours. The second component (component II) reached its peak activity three to five days following injection. Yamamoto, Skanderbeg, Zipursky and Israels (1965) and Ibrahim, Schwartz and Watson (1966) have produced evidence that component II probably begins on day 1, the initial part of its production being obscured by component I. The second peak corresponded in time to the maximal increment of radioactivity in red cell haem, when the red cells that had previously incorporated the label were emerging from the marrow. These observations implied a relationship

between component II and erythropoiesis.

Yamamoto, Skanderbeg, Zipursky and Israels (1965) and Ibrahim, Schwartz and Watson (1966) found that the excretion of early-labelled bilirubin in bile fistula dogs was markedly depressed after the first twelve to twenty-four hour peak following total body irradiation or hypertransfusion to depress erythropoiesis. Thus component II in bile fell along with a decrease in the synthesis of circulating red cell haem. Israels, Skanderbeg, Guyda, Zingg and Zipursky (1963) showed that depression of erythropoiesis in bile fistula dogs using busulfan was similarly followed by depression of component II. Conversely, in the presence of accelerated erythropoiesis following repeated bleeding in dogs, more glycine was utilized for both circulating haemin, and early-labelled bilirubin than in the normal. However, the percentage of the "precursor pool" diverted through the shunt was comparable to that in normal dogs (Israels, Skanderbeg, Guyda, Zingg and Zipursky, 1963; Ibrahim, Schwartz and Watson, 1966). Component I was relatively unaffected by this procedure. This amplified the earlier findings of Gray and Scott (1959) who had studied stercobilin labelling under similar experimental conditions.

Further studies by Israels, Yamamoto, Skanderbeg and Zipursky (1963a) and Yamamoto, Skanderbeg, Zipursky and Israels (1965) supported the multiple origin of early-labelled bilirubin. These studies employed 4-C^{14} delta-aminolevulinic acid (delta-ALA) as precursor material in place of glycine. It was shown that labelling first appeared in the

plasma bilirubin of human subjects within twenty minutes of intravenous injection and that maximal activity was reached by ninety minutes. Activity fell sharply to low levels at twenty-four hours and no second peak was observed. Labelled bilirubin in bile appeared more slowly and reached its peak at six hours. The percentage of injected counts appearing in circulating haem was much less than one percent. The finding that 4-C^{14} delta-ALA is an efficient precursor of bilirubin but is poorly incorporated into haemoglobin haem suggests that exogenous ALA gains access to the sites of haem synthesis more easily in hepatic than in erythroid cells. Although ALA is efficiently incorporated into haemoglobin by lysed reticulocytes, it is less well utilized by intact cells (Vavra, Mayer and Moore, 1964), suggesting that erythroid cells are relatively impermeable to ALA (Scott, 1955). For these reasons, 4-C^{14} delta-ALA became the tracer material of choice in studying the non erythropoietic component of shunt bilirubin. Several possible sources of shunt bilirubin may now be considered.

COMPONENT I (NON ERYTHROPOIETIC)

1) Turnover of tissue haem proteins, including catalase, myoglobin, the cytochromes, cytochrome oxidase, peroxidase, and tryptophane pyrrolase. Ibrahim, Schwartz and Watson (1966) have observed that about 20% of the injected radioactivity of 4-C^{14} delta-ALA appears in the bile early-labelled bilirubin of bile fistula dogs. The observed turnover

of label in the tissue haems of dogs (Schwartz and Cardinal, 1965) was consistent with their contribution of a significant portion of the C^{14} subsequently appearing in bilirubin.

Half-life determinations of liver catalase (Price, Sterling, Tarantola, Hartley and Rechcigl, 1962), tryptophane pyrrolase and cytochromes P-450 and b_5 (Berlin and Schimke, 1965 and Schmid, Marver and Hammaker, 1966), which probably represent the bulk of rapidly turning over tissue haemoproteins in the liver, revealed values of thirty hours, about one hour, less than twelve hours, and five days, respectively. Myoglobin, on the other hand, although it contains about five percent of the total body porphyrin (Drabkin, 1948) was found to have too long a half-life (Theorell, Beznak, Bonnichsen, Paul and Akeson, 1951) to be consistent with its acting as a significant source of early-labelled bilirubin. These authors observed that injected Fe^{59} was not significantly incorporated into myoglobin until more than a month following injection, much longer than the appearance time of shunt bilirubin. Thus, these haemoproteins, with the exception of myoglobin, may all have a sufficiently short half-life for them to function as early-labelled bilirubin sources. Complete quantitative data on the total turnover of these substances are not available; therefore, a final assessment of their role is not yet possible.

2) <u>Turnover of free haem in association with tissue haemoprotein synthesis.</u> There is a considerable body of indirect evidence that the haem and protein moieties of haemoproteins are synthesized

separately and subsequently assembled. Ingram and Winslow (1966) suggested that the "control point" observed in the synthesis of both the alpha and beta chains of haemoglobin may reflect the insertion of the haem group during peptide chain growth. Greengard and Feigelson (1963) found that the majority of tryptophane pyrrolase in adult rat liver exists in a haem-free inactive form which is activated through haem-binding in vivo by induction with tryptophane and in vitro by the addition of foetal liver cell sap which presumably contains available haem (Goldstein and Knox, 1963). Perhaps the most convincing evidence for the separate synthesis of haem and apoprotein were the findings by Baglioni (1966) and Felicetti, Colombo and Baglioni (1966) that: a) Fe^{59} which had been incorporated by reticulocyte polysomes was not released by puromycin which, however, was shown to release all protein chains; and b) in the presence of chelating agents which prevented haem synthesis, reticulocytes retained the ability to synthesize globin which was detectable by Sephadex chromatography.

If haem exists intracellularly as a free molecule, this moiety might be reasonably expected to undergo rapid catabolism; if present in sufficient quantity, such molecules could contribute to the early-labelled bilirubin.

The demonstration by Robinson, Owen, Flock and Schmid (1965) that the isolated perfused rat liver readily incorporates the radioactivity of 4-C^{14} delta-ALA or 2-C^{14} glycine into the early-labelled bilirubin was direct evidence for extraerythropoietic haem synthesis. The time sequence observed resembled that seen with in vivo experiments. White, Williams, Rother and Shafer (1966) demonstrated that a suspension of rat liver mitochondria in cell sap could mediate the conversion of 2-C^{14} glycine to haem

C^{14} and subsequently $C^{14}O$ and bilirubin-C^{14}. The short term tissue culture studies of Granick (1965) utilizing chick embryo liver cells suggested that active protein synthesis might be required at a specific point, early in the porphyrin biosynthetic pathway. Cultured chick liver cells were incubated in the presence of either glycine or delta-ALA and subsequently examined for porphyrin fluorescence. Puromycin and other inhibitors of protein synthesis prevented the appearance of fluorescence only in those cells not provided with delta-ALA, implying that unimpaired protein synthesis was essential to maintain delta-ALA synthetase activity in this system. With added delta-ALA porphyrin synthesis proceeded in a relatively normal fashion in spite of impaired protein synthesis. Protoporphyrin ferrochelatase activity was not determined in the system.

The rapid conversion of injected labelled protein-free haematin to bilirubin in vivo has been demonstrated by Pass, Schwartz and Watson (1945), London (1950), and Snyder and Schmid (1965). Snyder and Schmid (1965) recovered about seventy percent of the haematin radioactivity injected into rats as bile bilirubin within a few hours. These findings combined strongly suggest the existence of an extraerythropoietic pathway in which protein synthesis is not obligatory for the synthesis of haem from its simplest precursor and its subsequent catabolism to bilirubin and CO.

There are a number of reports in the literature describing clinical or experimental hyperbilirubinaemias which may be due to

the turnover of "redundant" free haem. A post surgical jaundice occuring in humans has been reported by several authors (Gachin, 1962; Caroli, Champeau and Desvignes, 1962; and Pichlmayr and Stich, 1962). The syndrome consisting of a postoperative hyperbilirubinaemia extending from about the first to the third postoperative day has been correlated with the duration and severity of surgery and the storage time of transfused blood. Conventional liver function tests, whose results are independent of protein synthesis were described as normal in these patients. Early-labelled bilirubin was not studied. In animals, McMaster, Broun and Rous (1923) observed that bile fistula dogs excrete an excess of bilirubin immediately after surgery.

Further, it has been suggested that in some cases of primary liver disease, the hyperbilirubinaemia may be at least in part caused by an overproduction of early-labelled bilirubin in the liver (Yamamoto, Skanderbeg, Zipursky and Israels, 1965; Kalk and Wildhert, 1955; and Siede, 1957). In the light of these observations it is tentatively suggested that hepatocellular hyperbilirubinaemia may result not only from an impairment of bilirubin excretion, but in some cases may at least in part result from hepatic overproduction of bilirubin.

Evidence against disproportionate synthesis of haematin and apoprotein has recently been presented by Marver, Tschudy and Perlroth (1966). These authors have presented evidence for a feedback control mechanism operating to coordinate the synthesis of haem and apoenzyme for the liver enzyme tryptophane pyrrolase. There is no evidence to support their contention that similar mechanisms may be operative in the biosynthesis of the other tissue haemoproteins. Baglioni (1966) produced evidence

that haem is not essential for globin synthesis. Greengard and Feigelson (1963) in work preceding that of Marver's group (1966) stated that the majority of tryptophane pyrrolase in adult rat liver exists in a haem-free inactive form which is activated through haem binding *in vivo* by induction with tryptophane and *in vitro* by addition of foetal liver homogenate cell sap (Goldstein and Knox, 1963). Goldstein and Knox (1963) further reported that foetal rat liver was devoid of tryptophane pyrrolase activity, although it did contain the activator of the adult apoenzyme, i.e., probably haem.

COMPONENT II (ERYTHROPOIETIC)

1) Catabolism of erythrocytes and/or their precursors prior to their release into the circulation. The findings of Israels, Skanderbeg, Guyda, Zingg and Zipursky (1963), Yamamoto, Skanderbeg, Zipursky and Israels (1965), and Ibrahim, Schwartz and Watson (1966) established a direct relationship between erythropoietic activity and component II of shunt bilirubin. This implied rapid turnover of a fraction of the intramedullary haem and/or haemoglobin. Destruction of a fraction of the body's red cell precursors in an intramedullary site probably provides at least a significant portion of this haem moiety. The absolute value of this fraction of haem might reasonably be supposed to bear a direct relationship to the total number of cells in the erythrocyte precursor pool; hence the previously described relationship of shunt bilirubin to stimulated versus depressed erythropoiesis.

The existence of pathological erythrocyte precursors with increased susceptibility to intramedullary destruction would cause an

increase in the fraction of abortive haem synthesis and hence in shunt bilirubin. Such a mechanism is thought to be operative in pernicious anaemia (London and West, 1950), congenital porphyria (Gray, Neuberger and Sneath, 1950), and thalassemia (Grinstein, Bannerman, Vavra and Moore, 1960). Because the increased marrow erythroid activity of these states is not accompanied by a commensurate increase in the "desired" end product, circulating haemoglobin, the term "ineffective erythropoiesis" has been applied. A simple illustration is presented to clarify the relationship between normal erythropoiesis, depressed erythropoiesis, stimulated erythropoiesis, and ineffective erythropoiesis in Figure 1 (page 11a), which consists of four flow diagrams, horizontally arranged depicting, respectively, the types of erythropoiesis indicated. The process of erythropoiesis is illustrated in three "vertical compartments" for each type, consisting of: 1) the haem precursor pool; 2) the intramedullary red cell precursor pool referred to as erythroblasts; and 3) the "products" which consist of shunt bilirubin and circulating haemoglobin. The proportion of each compartment participating in "effective" erythropoiesis with a product of circulating haemoglobin is unshaded while the shaded portions represent ineffective erythropoiesis.

It is obvious in the first three types of erythropoiesis (normal, depressed, and stimulated) that the proportion of the total resulting in ineffective erythropoiesis is the same although the absolute amount of shunt bilirubin produced varies greatly. In ineffective erythropoiesis the ratio is reversed, resulting in the predominance of ineffective as opposed to effective "product".

TYPES OF ERYTHROPOIESIS

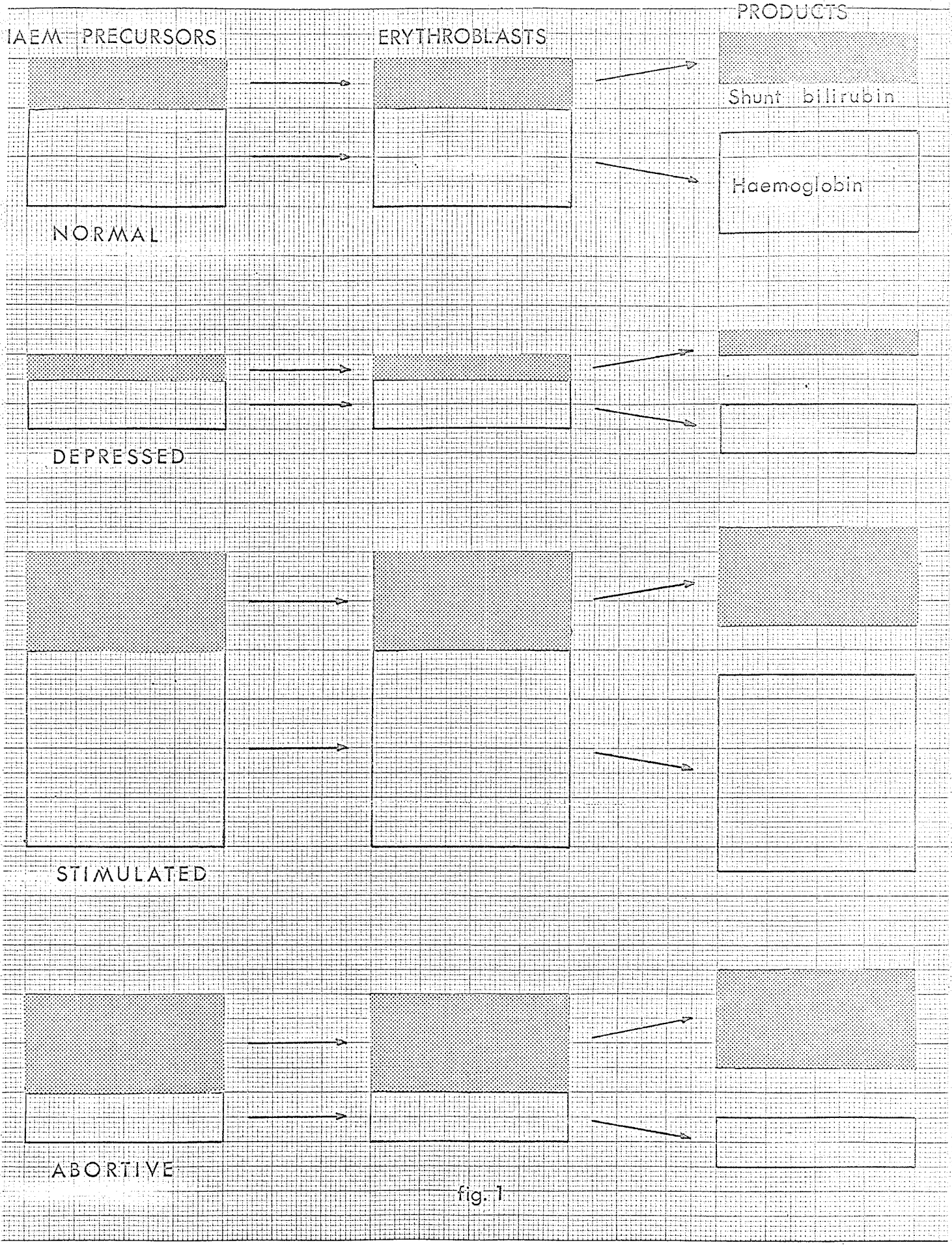


fig. 1

2) Redundant haem synthesis and/or loss of haem by erythrocyte precursors. The model described above relating erythropoiesis to shunt bilirubin is compatible with a rapidly turning over fraction consisting of haemoglobin and/or haem. The present state of knowledge regarding the role of free haem in bilirubin metabolism has been discussed in relation to the non erythropoietic fraction. Haemolysis in vivo is generally regarded as an "all-or-none" phenomenon in which consideration of intermediate stages of impaired membrane function is avoided. In reality, the red cell membrane may undergo various pathologic changes in permeability preceding ultimate destruction (Weed and Reed, 1966). These authors also state that red cell fragmentation may result in partial haemoglobin loss without necessarily implying immediate in vitro or in vivo haemolysis. Both of these mechanisms may be operative to varying degrees within the marrow, possibly in normal, and probably in some pathologic types of erythropoiesis, leading to rapid catabolism of the haemoglobin released.

3) Loss of haem and/or haemoglobin from erythrocyte precursors accompanying extrusion of the normoblast nucleus. This was postulated as a possible source by Bessis, Breton-Gorius and Thiery (1961). No conclusive data are available with regard to the significance of this mechanism in mammals. Ducks, however, are known to have circulating nucleated erythrocytes with a pattern of shunt bilirubin similar to that of mammals, including component II (Israels, Novak, Foerster and Zipursky, 1966). From these findings it is evident that nuclear extrusion by normoblasts

is not a prerequisite for the formation of component II of shunt bilirubin.

HAEM PRECURSORS AND OTHER SOURCES

There is little information in the literature to suggest that bilirubin formation can occur in the absence of haem synthesis. Various bilirubinoid pigments are characteristic of lower forms of life. In some these have been found without known relationship to haems or haemoglobin and have been believed to be formed de novo. The best examples of these are the bilirubinoid protein complexes known as phycoerythrin and phycocyanin known to participate in the photosynthetic processes of red or blue marine algae (Watson, 1965). The absorption of light by the red-violet pigment is much greater than that of chlorophyll. The possibility that a haem is involved in this pathway has been recently suggested but remains to be definitely established (Nichols and Borograd, 1962 and O'hEocha, 1965).

There are important objections, in higher forms particularly, to the concept of bilirubin formation in the absence of haem synthesis. It is believed that the proposed tetrapyrrylmethane intermediary between porphobilinogen and uroporphyrinogen cyclizes immediately. Conceivably, a fraction of this might be oxidized, preventing cyclization and giving rise to a urobilirubinoid pigment. However, according to recent concepts of uroporphyrinogen III formation by Mathewson and Corwin (1961) and Bullock, Johnson, Markham and Shaw (1958) a compound of this type would probably lack, if the ring closed, what would be the gamma rather than the alpha methene bridge (Petryka, Nicholson and Gray, 1962 and Pullman

and Perrault, 1959). It is well known that the enzymatic formation of bile pigment from haem is due to loss of the alpha methene bridge as carbon monoxide (Petryka, Nicholson and Gray, 1962; Pullman and Perrault, 1959; and Sjostrand, 1949). This is highly specific in nature. If a urobilirubinoid pigment were formed from tetrapyrrylmethane, it should be readily distinguishable from the bilirubinoid pigment formed from haem. Moreover, the bilirubinoid of the red algae is of nine alpha type (Lemberg and Bader, 1933), pointing strongly to a haem derivation. On the other hand, an unpublished observation by Petryka (Watson, 1965) reveals that early-labelled bilirubin of dog bile following injection of 4-C^{14} delta-ALA, upon oxidation, yields a small proportion of a C^{14} labelled carboxy pyrrol which could not be derived from bilirubin 9, alpha. This does not prove that this bilirubin isomer is not haem-derived as bile pigment formed by the coupled oxidation of haem in vitro is a mixture of isomers rather than the highly specific loss of the alpha bridge seen in vivo (Petryka, Nicholson and Gray, 1962).

It has not been established whether the enzymatic conversion of tissue haems is strictly specific as it is for haemoglobin. The finding of a urobilirubin in bile would provide significant evidence for the synthesis of a porphyrin-derived shunt bilirubin bypassing haem. In the present state of knowledge, therefore, the formation of bile pigments by a pathway not involving haem degradation would appear unlikely but not impossible.

At this point, the available information may be interpreted as indicating multiple sources of origin for shunt bilirubin. The degradation

of haem of both erythropoietic and non erythropoietic nature appear to be obligatory in the process. The role of protein-association of these haems is not established.

The primary objective of the present investigations has been to study the non erythropoietic fraction of shunt bilirubin and its relationship to tissue haem synthesis under normal conditions and conditions of altered tissue haem synthesis. Studies have been carried out on Sprague-Dawley rats.

CHAPTER II

THE RELATIONSHIP OF NON ERYTHROPOIETIC SHUNT BILIRUBIN TO TISSUE HAEM SYNTHESIS IN THE LIVER AND KIDNEY IN "NORMAL RATS"

A. INTRODUCTION

Schwartz and Cardinal (1965) and Schwartz, Ibrahim and Watson (1966) have studied the relationship of labelling of shunt bilirubin and non haemoglobin haems in liver, kidney and other tissues using 4-C^{14} delta-ALA as precursor material in dogs. Robinson, Owen, Flock and Schmid (1965) demonstrated the de novo synthesis of labelled bilirubin from 2-C^{14} glycine or 4-C^{14} delta-ALA in isolated rat liver preparations in the absence of erythropoiesis. These findings have been confirmed in unpublished data obtained by Dr. W. Novak in our laboratory.

The objective of this series of experiments was to establish the quantitative relationship in normal rats between the uptake of radioactivity from injected 4-C^{14} delta-ALA into early-labelled bilirubin and C^{14} turnover in the tissue haems of liver and kidneys. The data to be presented were obtained in part with collaboration of Dr. Novak.

B. MATERIALS AND METHODS

1) General Comments. Male Sprague-Dawley rats weighing 300 - 400 grams were used throughout these experiments. They received a normal laboratory diet and were permitted free access to water. In all cases

the precursor material used was 4-C^{14} delta-ALA of specific activity between 25 - 30 mc/mM or .15 - .2 uc/ugm. Intravenous injections were carried out through the tail vein in a dosage of 1 microcurie which contained five to seven micrograms. Subsequently, animals were once again permitted access to food and water ad lib until termination of the experiment.

2) Bile Collections. On the morning of each experiment, the animals were fasted for several hours to facilitate surgery. The common bile duct was easily exposed by means of a midline abdominal incision and ligated close to its junction with the duodenum. After several minutes, dilation of the duct was sufficient to permit placement of a small transverse incision about .5 cm above the ligature, enabling insertion of a fine polyethylene cannula into the common duct. A few minutes later, when adequate bile flow through the cannula was evident, a ligature was placed around the duct just above the point of insertion of the cannula. The intraluminal end of the cannula was carefully placed within the common duct itself and was withdrawn an appropriate distance if it was found to have ascended to the level of the hepatic tributaries. The ligature was then firmly secured to prevent further changes in position of the end of the catheter and ensure total collections. After haemostasis was accomplished, the incision was closed around the biliary catheter with polyethylene or silk sutures. The animal was then placed in a loose restraining cage and permitted 18 to 24 hours tc recover from the acute effects of anaesthesia and surgery.

Ether was employed as the anaesthetic agent in all experiments. In some cases, hydration of the animals was supplemented with subcutaneous injections of small volumes of normal saline. Although techniques of strict asepsis were not empolyed, purulent peritonitis was not demonstrated in a number of post mortem examinations and only a small number of these animals were found to have suture infections.

Eighteen to twenty-four hours after completion of surgery the general clinical condition and bile flow of the animals was reassessed. Those who appeared to be in poor condition or had a flow of less than 0.5 cc per hour were excluded from the experiment. Such animals formed only a small minority of the total number undergoing surgery.

Injections of delta-ALA were carried out in most cases without anaesthesia although extreme irritability in a few animals necessitated the use of light ether anaesthesia. Each rat received one microcurie of delta-ALA by intravenous tail vein injection. Collections of bile were made in semi-darkness and with a few grains of ascorbic acid in each collecting vessel to prevent oxidation of the bilirubin to biliverdin. Collecting vessels were changed at appropriate intervals to permit sequential determinations of C^{14} incorporation over a 24-hour period following injection. At this time, the experiment was usually terminated and the animal sacrificed.

The interval bile samples collected were immediately frozen pending bilirubin extraction which was always carried out within two weeks. In a few animals in whom slight haematobilia was observed, bile samples were immediately centrifuged to remove red blood cells prior to freezing.

3) Bilirubin extraction. Prior to bilirubin extraction, a quantitative bilirubin estimation was performed on each aliquot by a micromethod modification of the method of Malloy and Evelyn (1937). Modification of the reagents was as follows:

Reagents

Solution A: 3.0 gm sulfanilic acid dissolved in 15 cc concentrated HCl and diluted to 1000 cc with doubly distilled water.

Solution B: 1% sodium nitrite.

Diazo reagent: 10.0 ml of solution A and 0.5 ml, solution B.

Diazo blank: as in macro method.

Mixtures

Test	Blank
1.2 ml absolute methanol	1.2 ml absolute methanol
0.2 ml diazo reagent	0.2 ml diazo blank
1.0 ml diluted bilirubin or bile	1.0 ml diluted bilirubin or bile

Procedure: Mix well and read at 540 mu, slit width 0.05 mm, and suitable sensitivity on Beckman DU spectrophotometer after standing for 5 minutes.

Standard curves were prepared with weighed amounts of commercially available bilirubin*(Jendrassik and Grof, 1938). Bilirubin concentration of the bile in milligrams percent was determined from the prepared standard curve. Bilirubin was then extracted and crystallized from each sample using unlabelled carrier crystalline or bile bilirubin where required by the method of Ostrow, Hammaker and Schmid (1961). Following the initial

* Nutritional Biochemicals. This material is completely chloroform soluble.

crystallization, the bilirubin was redissolved in chloroform and further purified by alumina column chromatography (Schwartz and Watson, 1942 and Israels, Skanderbeg, Guyda, Zingg and Zipursky, 1963a). The purity of the resultant crystalline bilirubin from contaminants such as glucuronide was supported by the demonstration (Israels, Skanderbeg, Guyda, Zingg and Zipursky, 1963a) that it underwent no significant change in specific activity after repeated chromatography on the reverse phase kieselguhr system of Cole and Lathe (1953).

The final pure bilirubin was once again redissolved in chloroform and bilirubin concentration quantitated by the micromethod modification of the diazo reaction (Malloy and Evelyn, 1937). Aliquots of 0.05 to 0.1 milligrams were then plated in duplicate at infinite thinness and counted to determine C^{14} activity in a Nuclear-Chicago Gas Flow Counter. Specific activity of the bile bilirubin was calculated by correcting for the amount of added "cold" carrier. With this information and the bilirubin concentration and volume of each bile sample, the bilirubin-C^{14} excretion for each time interval was determined and plotted as a percentage of total injected isotope.

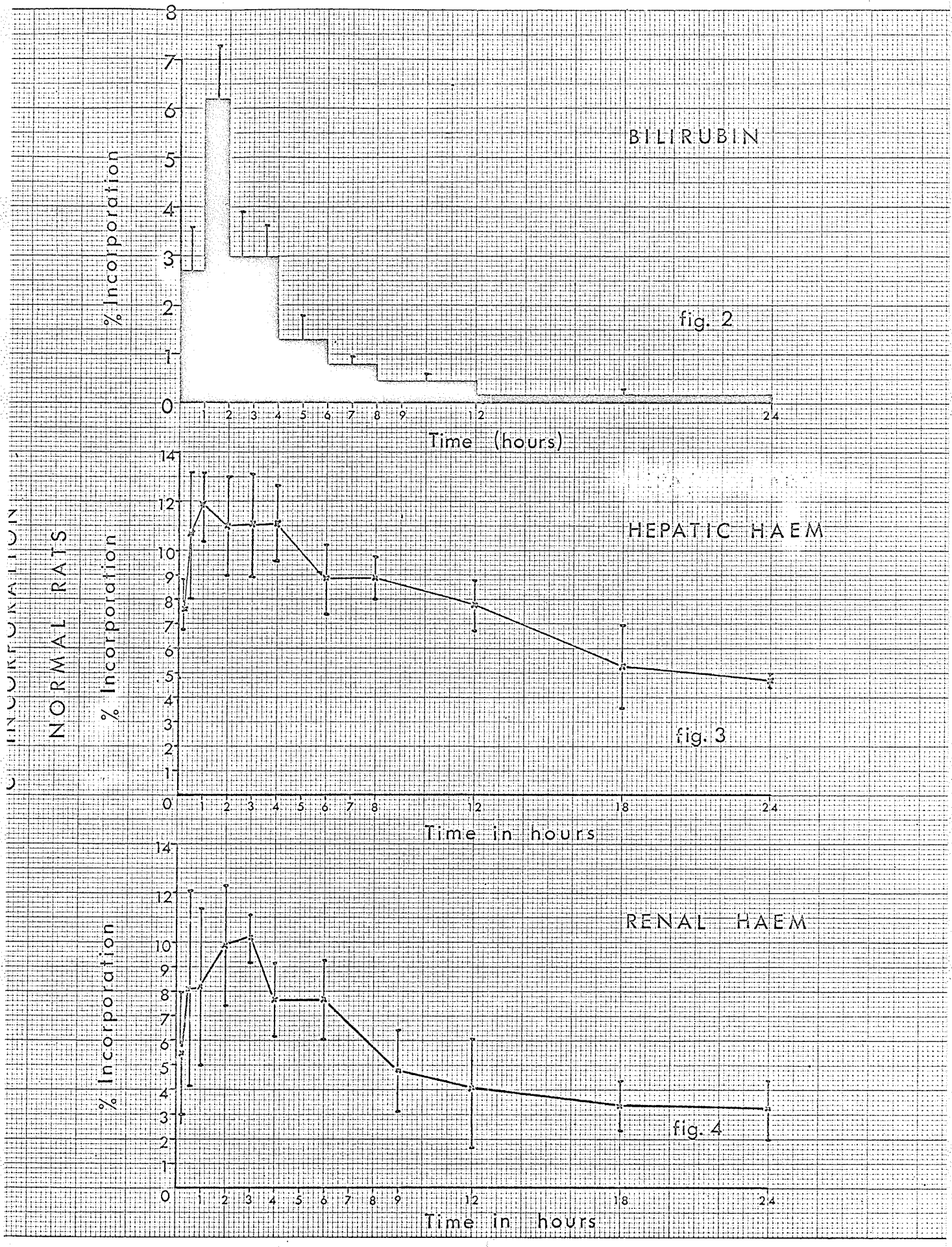
4) Haem determinations. These animals received a tail vein injection of one microcurie of delta-ALA, using light ether anaesthesia when necessary. At suitable intervals up to 24 hours following injection, groups of approximately eight animals were sacrificed by a blow on the head. Within a period not exceeding five minutes, livers and kidneys were removed, perfused with cold normal saline to remove blood, and frozen pending haem extraction. This was always accomplished within one week of the experiment.

Immediately after thawing, the liver and kidneys of each rat were separately homogenized in 4 - 5 volumes of double distilled water, first in a Waring blender and finally in a Potter Elvehjem homogenizer using a teflon pestle. One hundred milligrams of carrier haem in haemoglobin prepared from human polycythemic blood was added to each of the above homogenates and haem was extracted by the method of Labbe and Nishida (1957). Specific activity of the haem isolated by this method was not altered by pyridine recrystallization as described by Shemin, London and Rittenberg (1950).

The haem crystals were dissolved in 0.1 N NaOH and aliquots of 1 milligram by weight were plated in duplicate at infinite thinness and counted to determine C^{14} activity in a Nuclear-Chicago Gas Flow Counter. In this case, the amount of endogenous haem present in the system and, therefore, its specific activity could not be determined. However, the total liver nonhaemoglobin haem is small compared to the carrier added. On the basis of this assumption, the specific activity of the isolated haem was used to calculate the total nonhaemoglobin haem C^{14} content of each organ. Results were plotted and expressed as a percentage of the injected isotope at each point in time.

C. RESULTS

1) Early-labelled bilirubin. Figure 2 is a bar graph depicting the percentage of the injected C^{14} excreted in bile bilirubin during the time intervals indicated following the intravenous injection of one microcurie of 4-C^{14} delta-ALA. The vertical lines above the centre of


BILIRUBIN
% Incorporation
Time (hours)
fig. 2
HEPATIC HAEM
% Incorporation
Time in hours
fig. 3
RENAL HAEM
% Incorporation
Time in hours
fig. 4
NORMAL RATS

each bar represent the standard deviation calculated for a group of six rats.

During the first hour, an average of 2.7 percent of the injected counts were incorporated. Peak incorporation was observed in all of the six rats during the second hour of collection. The bilirubin isotope content thereafter gradually declined to a level of about 0.33 percent per hour between the twelfth and twenty-fourth hours. The percentage of isotope incorporated over the total twenty-four hour period was calculated as 22.71 percent with a standard deviation of 0.96 percent. Bile bilirubin concentration and volume excreted remained quite constant throughout the twenty-four hour period.

2) Liver haems. Figure 3 represents the percentage of injected C^{14} incorporated in rat liver at the points in time indicated following intravenous injection of 4-C^{14} delta-ALA. The X's represent the mean values calculated for the rats in each group and the vertical lines through the X's represent the standard deviations calculated from a number of observations at each point. The number of observations made were: three at one-quarter hour; seven at one-half hour; six each at one, two, three, and four hours; five at six hours; four at eight and twelve hours; and three and two at eighteen and twenty-four hours, respectively.

By fifteen minutes, 7.5 percent of the injected counts had been incorporated in hepatic haem. Peak incorporation was observed at one hour. Following this, a plateau of isotope incorporation extended to the fourth hour, followed by a gradual decline to about 4.7 percent by the

twenty-fourth hour. Thus at peak activity, liver haem contained about twelve percent of the injected counts, but by twenty-four hours less than five percent remained; seven percent of the injected C^{14} having apparently been lost from hepatic haem during the twenty-four hour period.

3) Renal haems. Figure 4 represents the incorporation of injected C^{14} into renal haem in the same way that hepatic haem is depicted by Figure 3. The number of observations made for each point were: seven at one-quarter hour; five at one-half hour; six each at hours one, two, three, four and six; and three each at hours nine, twelve, eighteen and twenty-four. Fifteen minutes after injection, renal haem contained 5.5 percent of the injected activity. Peak incorporation was seen at three hours at which point ten percent of the injected counts had been incorporated. A gradual decline in activity was then observed to a level of about three percent by twenty-four hours. Thus, at peak activity, renal haem contained about ten percent of the injected label but by twenty-four hours, only three percent remained; seven percent of the injected label having apparently been lost by renal haem in the twenty-four hour period.

D. DISCUSSION

Figure 5 consists of superimposed cumulative bar graphs plotting: a) the cumulative excretion of injected C^{14} as bilirubin; and b) the cumulative loss of C^{14} from the tissue haems studied. The graph depicting tissue haem C^{14} loss is divided into renal and hepatic components as illustrated.

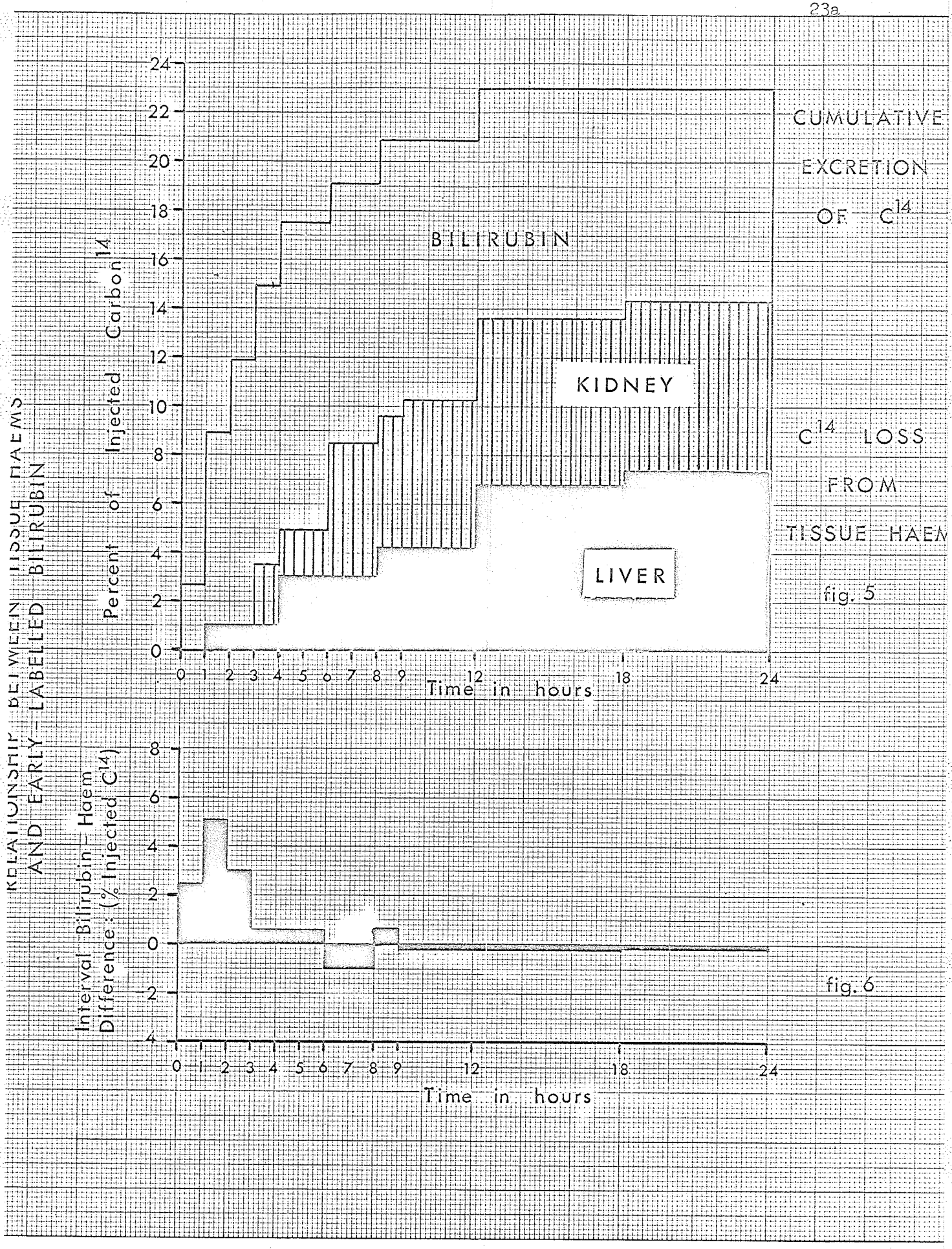


fig. 5

C14 LOSS FROM TISSUE HAEM

fig. 6

Over the twenty-four hour period, approximately twenty-three percent of the injected C^{14} was excreted as bile bilirubin. The loss of C^{14} from renal and hepatic haems over the same period appeared to total only 14.3 percent, or a difference of about 8.7 percent.

One other feature illustrated by Figure 5 is to be emphasized. Examination of the early part of the graph shows that in the first hour, labelled bilirubin had appeared while the activity in both renal and hepatic haems was still rising. The greatest increment in bilirubin C^{14} was seen during the second hour at a time when loss of activity from tissue haems was still minimal. Following hour three, increments in bilirubin C^{14} excretion appeared to be approximately paralleled by loss of C^{14} from tissue haems.

Figure 6 was constructed in order to more clearly illustrate the relationship between loss of C^{14} from tissue haems and the appearance of C^{14} in bilirubin. The increments of C^{14} in bilirubin and the combined loss of C^{14} from tissue haems were calculated for each interval. Haem loss for each interval was then subtracted from bilirubin increment and the resultant value was calculated on a "per hour" basis. Thus excretions of bilirubin C^{14} in excess of loss of C^{14} from tissue haem are plotted as positive values whereas the reverse result in negative values. The graph thus constructed (Figure 6) indicates that during the first six hours, 12.4 percent of the injected C^{14} recovered from bilirubin appears to be "unaccounted for" by loss of C^{14} from tissue haems. Following this time, primarily negative values are recorded, as would be expected if the relationship were such that the labelling of bilirubin were preceded by

quantitative loss of label from a uniformly turning over pool of tissue haems.

In order to interpret the observed relationship it must be borne in mind that, as discussed in Chapter I, the tissue haem pool, unlike the erythropoietic haem pool, is heterogeneous. As a result, turnover times for the various components differ and the values plotted in Figures 2 and 3 are actually composites resulting from simultaneous synthesis and degradation of haems possessing different turnover times. In view of this, it is likely that some loss of C^{14} from very rapidly turning over haems occurs during the early periods of label increment but that this is overshadowed by simultaneous label incorporation in more slowly turning over haems. Therefore, two artefacts are inherent in the recorded observations, particularly during the period of haem-labelling increment: 1) early loss of label from short-lived haems is overshadowed; 2) the observed values for peak labelling likely underestimate the total incorporation of C^{14} into all haems because of the prior loss of some C^{14} from the short-lived haems.

In the light of these considerations, the bilirubin-haem difference observed during the first six hours may well be only an apparent one, undetected loss of C^{14} from short-lived haems contributing some or all of the 12.4 percent deficit. The predominantly negative deflection in the graph after the sixth hour is consistent with loss of label from a fairly uniform haem pool preceding the appearance of this label in bilirubin.

CHAPTER III

THE RELATIONSHIP OF NON ERYTHROPOIETIC SHUNT BILIRUBIN TO TISSUE HAEM SYNTHESIS IN LIVER AND KIDNEY

A. INTRODUCTION

The second chapter documented the relationship between the turnover of C^{14} in liver and kidney haem and its appearance in the bile bilirubin of normal rats. Further elucidation of this relationship required observations under varying experimental conditions designed to alter and thereby further elucidate the relationship.

1) Biliary obstruction. Biliary obstruction was shown by Aronsen and Haeger-Aronsen (1963) to result in a depression of heptaic catalase activity in rabbits and dogs. Although the exact fraction of liver tissue haem existing as catalase haemoprotein has not been determined, Higashi and Peters (1963) using a combination of immunochemical and enzymatic techniques concluded that rat liver contains about 1550 micrograms of tissue catalase per gram wet tissue weight. Bonnichsen (1948) estimated the average molar concentration of haem in guinea pig liver catalase as 3 moles of haem per mole of catalase. Extrapolating this ratio to the rat yielded a calculated value of about 13 micrograms of catalase haem per gram of wet liver tissue or about 195 micrograms per average fifteen-gram liver. Using this value and the thirty-hour hepatic catalase half-life determined by Price, Sterling, Tarantola and Rechcigl (1962), one may calculate a daily turnover of 80 micrograms of haem from this source, about 6.7 percent of the total daily bilirubin excretion.

Stotz (1939) and Potter and Dubois (1942) estimated the concentration of cytochrome c, another quantitatively important haemoprotein, in rat liver. The values obtained (68 micrograms per gram wet tissue by Stotz and 90 micrograms per gram wet tissue by Potter and Dubois) are in reasonable agreement and yield an average of 80 micrograms per gram of liver. With a molar concentration of one haem per molecule of enzyme and a molecular weight of 15,600 for the enzyme (Cohn and Edsall, 1950) a liver cytochrome c haem content of about 3 micrograms per gram wet tissue may be calculated. This is equal to about twenty-five percent of the liver catalase-haem content. Turnover data for these proteins was presented in Chapter I.

With these data it seemed reasonable to implicate catalase as a significant component of hepatic tissue haem. It was, therefore, undertaken to determine the relationship of hepatic haem to shunt bilirubin in the presence of biliary obstruction, a manoeuvre known to depress hepatic catalase activity.

2) Nephrectomy. The turnover of C^{14} label in renal tissue haem during the twenty-four hours following injection of delta-ALA as described in the preceding chapter was approximately equal in magnitude to that of liver. It was, therefore, decided to determine the effects of ablating this haem pool on the quantitative excretion of C^{14} in early bilirubin.

Complicating features inherent in this experiment were due to characteristics of the normal metabolism of delta-ALA in rats (Berlin, Neuberger and Scott, 1956 and 1956a). Between one-third and two-thirds of injected C^{14} or N^{15}-labelled delta-ALA was recovered in the urine within three hours, about half as unaltered delta-ALA. Bilateral nephrectomy in

eliminating this route of tracer loss should, theoretically, cause a prolongation of exposure of the remaining haem biosynthetic tissues to injected 4-C^{14} delta-ALA. The experiments were performed with these qualifying factors in mind.

3) Radiation. Israels, Skanderbeg, Guyda, Zingg and Zipursky (1963) and Israels, Yamamoto, Skanderbeg and Zipursky (1963) observed an increase in the specific activity of component I of the early-labelled bilirubin in bile-fistula dogs following 700 R of total body radiation. The increased specific activity was seen during the first two days following injection of 2-C^{14} glycine, while component II was greatly reduced as was the subsequent incorporation of C^{14} into circulating haem. More recently, Goldfeder and Miller (1963) described alterations in the subcellular distribution of catalase activity in mouse tumors within one hour following as little as 50 R of radiation. This consisted of a shift of catalatic activity from the mitochondria to the cell sap fraction without change in total cytoplasmic catalase content at doses up to 10,000 R. The authors suggested that radio-resistance is related to the capacity of organisms to transfer catalase into the soluble subcellular fraction, presumably to eliminate the hydrogen peroxide generated during radiation.

These effects of radiation on component I of bilirubin and catalase prompted a study of the hepatic haem-shunt bilirubin relationship in radiated rats.

4) Amino-triazole (A.T.). Hepatic and renal, but not blood, catalase activity is rapidly reduced to about ten percent of control

values following the intraperitoneal injection of 3-amino-1, 2, 4-triazole (A.T.) in rats (Heim, Appleman and Pyfrom, 1955). Margoliash, Novogrodsky and Schejter (1960) demonstrated that this inhibition is due to an irreversible binding with catalase. They also showed that a reaction mixture of crystalline enzyme and hydrogen peroxide could be inhibited by other substances with a basic structure of $N\text{-}NH\text{-}C(NH_2)\text{-}R$.

Since the mechanism of degradation of the haem moiety of the enzyme-inhibitor complex was unknown, it was of interest to observe the effect of A.T. administration on the pattern of early-bilirubin excretion. If complex formation led to enhanced destruction of the molecule with release of haem, one might expect a secondary peak in bilirubin-C^{14} excretion shortly after the injection of A.T.

5) Phenobarbital. Schmid, Marver and Hammaker (1966) reported that treatment of Sprague-Dawley rats with phenobarbital and starvation produced increases in: a) the incorporation of 2-C^{14} glycine into early-labelled bilirubin; and b) hepatic content of the microsomal cytochromes, P-450 and b_5, by spectrophotometric determinations. The mitochondrial cytochromes, catalase, and tryptophane pyrrolase were not significantly altered. The authors concluded that cytochrome P-450 has a half-life of less than twelve hours, considerably shorter than that of cytochrome b_5 or catalase and that it is, therefore, possibly a major source of component I of shunt bilirubin.

Granick (1965) had previously noted that phenobarbital administration resulted in delta-ALA synthetase induction. The possibility

that the increase in early-bilirubin labelling reported by Schmid and his co-workers was due to a non-specific activation of the haem biosynthetic pathway by induction of delta-ALA synthetase could not be eliminated using glycine as the precursor material. Therefore, 4-C^{14} delta-ALA was used as precursor material in our system. Hepatic microsomal cytochrome P-450 determinations were performed in our laboratory by Dr. B. Schacter. Both bilirubin and cytochrome experiments were carried out using various phenobarbital dosage schedules to determine a dose-response relationship.

6) Allylisopropylacetamide (A.I.A.). Treatment of rats with A.I.A. or its congener, Sedormid (allylisopropylcarbamide), has several effects on the synthesis of hepatic haem and haemoproteins: a) induction of delta-ALA synthetase (Granick, 1963); b) augmentation of overall haem synthesis (Schmid, Figen and Schwartz, 1955 and Lottsfeld, Labbe and Aldrich, 1961); and c) inhibition of catalase synthesis (Schmid, Figen and Schwartz, 1955 and Price, Sterling, Tarantola, Hartley and Rechcigl, 1962). Robinson, Tsong, Brown and Schmid (1966) reported that intravenous infusion of A.I.A. in a single Sprague-Dawley rat had no significant effect on the pattern of shunt bilirubin labelling following injection of 2-C^{14} glycine. Several features of their data, however, may be open to criticism. Firstly, the value obtained for the single experimental rat was actually at the upper limit of the range observed in their control animals; secondly, their conclusion is based on a single observation in the presence of a relatively large standard error in the control group;

thirdly, only glycine and not delta-ALA was employed as precursor material; and fourthly, the bile collection intervals were too great (0 - 3.5 hours, and 3.5 - 60 hours) to depict early-bilirubin kinetics with adequate precision. With these criticisms in mind, it was felt that further observations on the effects of A.I.A. on shunt bilirubin labelling were warranted.

B. MATERIALS AND METHODS

1) General Comments. Male Sprague-Dawley rats weighing 300 - 400 grams each were employed throughout the following experiments. Surgery for bile fistulae, tracer injections, bile collections, and bilirubin and haem extractions, isotope content determinations, and calculation procedures employed in this chapter were the same as those in Chapter II. The various manoeuvres used to induce alterations in the relationships observed in Chapter II will be described under their respective headings.

2) Biliary Obstruction. Complete extrahepatic biliary obstruction was established in the following manner: the common bile duct was exposed as in Chapter II and a tight, occlusive silk ligature was placed around it just proximal to its insertion into the duodenum. After a few minutes' delay to assure complete obstruction, the abdomen was closed and the animal replaced in its cage with access to food and water ad lib until termination of the experiment. The problem of

excessive bleeding which arose during the subsequent operative procedure to establish the bile fistula was eliminated by the subcutaneous injection of 0.2 to 0.3 milligrams of Aqua Mephyton (vitamin K_1) in all animals twenty-four hours following the first operative procedure.

Forty to forty-eight hours following bile duct occlusion, bile fistulae were established in all animals and 4-C^{14} delta-ALA injections and haem and bilirubin determinations were carried out within three hours. The twenty-four hour "recovery" period between surgery and injection used in Chapter II was omitted in this group in order to observe the acute effects of biliary obstruction.

3) Nephrectomy. Rats in this group were subjected to bilateral nephrectomy and bile duct cannulation at a single operation through a midline abdominal incision. The kidneys were first exposed and excised after placing a tight double silk ligature around each pedicle. The bile fistula was then established in the usual fashion. Because of the limited period of viability of these animals, about thirty-six hours post nephrectomy, delta-ALA injection was always carried out within three hours after surgery. Bilirubin collections and determinations were carried out as in Chapter II.

4) Radiation. The rats in this group were subjected to total body irradiation of either 700 or 1000 R. Groups of four to six animals were placed in a cardboard container of convenient size to facilitate administration of the radiation. X-ray dosage was delivered by a

Westinghouse Quadrocondex deep therapy X-ray unit utilizing a filter of 1 millimeter copper and 1 millimeter aluminum. Tube-surface distance employed was 112.5 cm with a surface dose rate of 20.1 R per minute. Both dosage levels consistently caused leukopenia and thrombocytopenia commencing four days following treatment but at the termination of a ten day observation period of several animals, only the 1000 R dose was observed to be lethal.

Bile duct fistulae were established in groups of the animals receiving 700 R at daily intervals from one to seven days following irradiation. Only a few animals received 1000 R and all of these underwent bile fistula surgery on the first day following irradiation. Once again, the twenty-four hour "recovery" period between surgery and delta-ALA injection was omitted in order to observe the acute effects of the procedure. Haem and bilirubin isolations were carried out as in Chapter II. There was no gross difference between these animals and the controls with regard to post surgical infection.

5) Amino-triazole (A.T.). Biliary fistulae were established. Following a twenty to twenty-four hour "recovery" period, one microcurie of 4-C^{14} delta-ALA was injected intravenously and bile collections were begun. 3-amino-1, 2, 4-triazole (50 mg/ml in normal saline) in a dose of one gram per kilogram was injected subcutaneously exactly twelve hours after injection of the tracer. Bile was collected at hourly intervals for three hours after A.T. injection to permit precise observations to be made. Subsequent collections were made at the usual intervals. Haem

determinations were not done in this series.

6) Phenobarbital. Groups of rats were given five consecutive daily subcutaneous injections of sodium phenobarbital ranging in dosage from zero milligrams per kilogram per day (controls received saline) to eighty milligrams per kilogram per day. A number of the animals were deprived of food from the fourth day of injection onward. On the sixth day bile fistulae were established in all animals and a sixth dose of sodium phenobarbital was administered following recovery from anaesthesia. Either one microcurie of 4-C^{14} delta-ALA, or twenty or one hundred microcuries of 2-C^{14} glycine was injected intravenously on the seventh day and bile collections and bilirubin extractions were carried out.

A second group of rats similarly treated with sodium phenobarbital were sacrificed on the sixth day without bile fistulae. Determinations of hepatic cytochrome P-450 were carried out in our laboratory by Dr. B. Schacter using the method of Omura and Sato (1964).

Three other animals were given 80 milligrams per kilogram per day of phenobarbital for five days and deprived of food from day four onward. On the sixth day they received one microcurie of 4-C^{14} delta-ALA intravenously and were sacrificed one hour later. Hepatic haem incorporation at one hour post injection was determined.

7) Allylisopropylacetamide (A.I.A.). These animals were given 2 grams per kilogram of A.I.A. (100 mg/ml in propylene glycol) in ten divided subcutaneous injections over a period of five days prior to surgery. Injections of equivalent amounts of propylene glycol alone in control

animals produced no deleterious effects. Injections of delta-ALA were carried out within a few hours of the establishment of bile fistulae and bilirubin determinations were performed as in Chapter II.

C. RESULTS

1) Biliary Obstruction.

a) Bilirubin. Figure 7 (page 35a) consists of two superimposed bar graphs comparing 4-C^{14} delta-ALA incorporation in the control animals of Chapter II (shaded) and the biliary obstructed animals (unshaded). The abscissa represents hours after isotope injection and the ordinate, the percentage of injected radioactivity excreted per hour in bile bilirubin. The vertical lines through the histogram bars represent the standard errors calculated from at least six "biliary obstructed" rats for each bile collection interval.

During the first two hours, isotope incorporation in the bilirubin of control rats significantly exceeded that of biliary obstructed rats. Subsequently and particularly from the second to the twelfth hour, much more isotope was incorporated into the bilirubin of biliary obstructed rats than in controls. It will be recalled that the twenty-four hour total incorporation of the control animals was 22.71 percent with a standard error of 0.96 percent. The biliary obstructed rats incorporated 52.11 $\pm$ 13.5 percent in twenty-four hours. The difference observed was

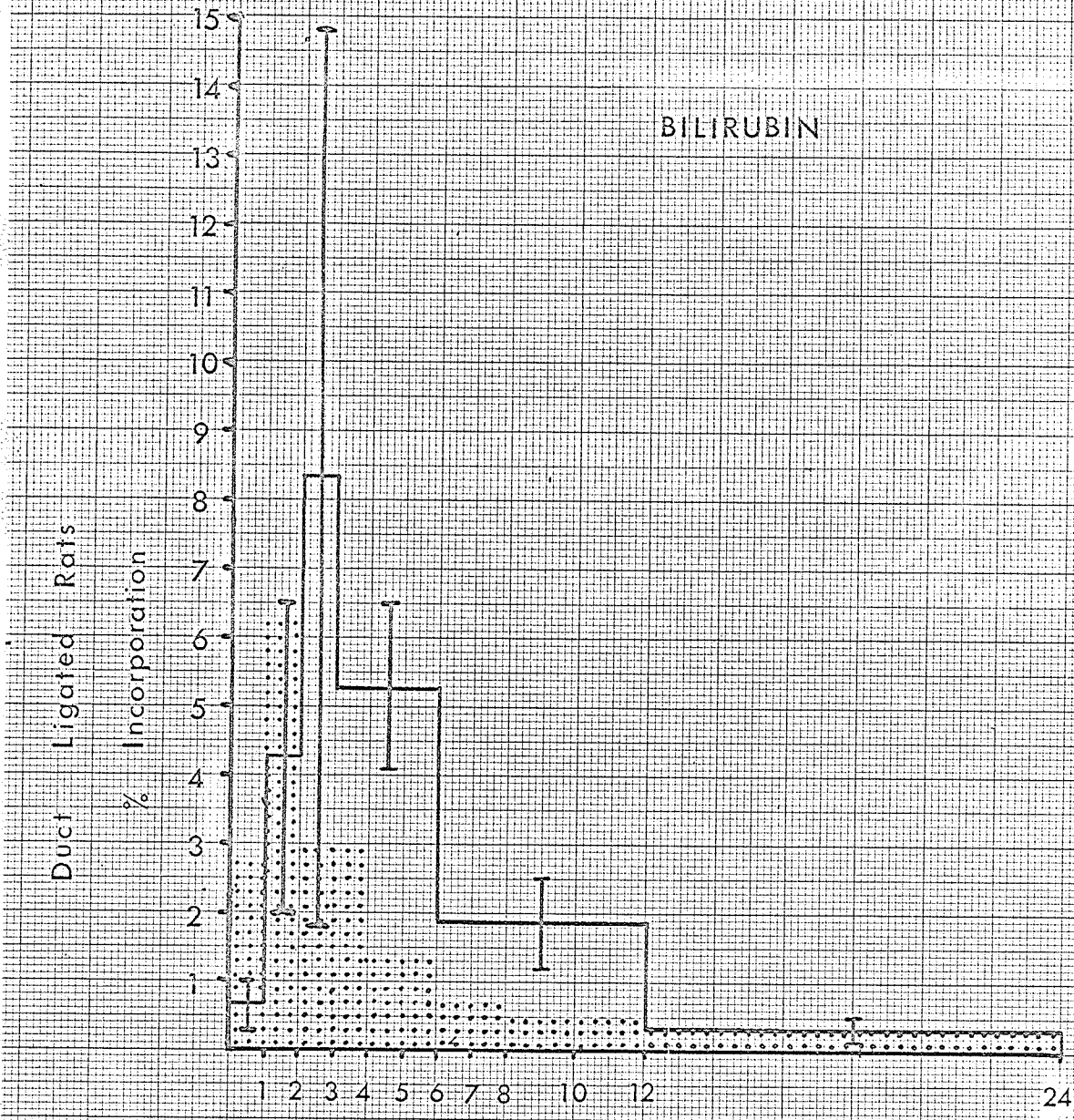

BILIRUBIN
% Incorporation
Duct Ligated Rats
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 10 12 24

Figure 7

significant by the Student T test to a level of $P < .01$.

The total twenty-four hour bilirubin excretions were calculated from the bile volumes and bilirubin concentrations and were found to be in the control and biliary obstructed groups, respectively: 1.28 ± 0.35 milligrams and 4.01 ± 0.94 milligrams. This difference was significant to a level of $P < .01$ by the Student T test. The bilirubin excretions were comparable in the two groups after the twelfth hour, suggesting that the bilirubin load retained by the biliary obstructed rats was cleared in the first twelve hours.

b) Haem. Figures 8 and 9 (page 36a) depict isotope incorporation into hepatic and renal haem. The abscissa in both cases represents hours after injection of 4-C^{14} delta-ALA and the ordinate is the percent of injected isotope incorporated into haem. The shaded areas in both graphs represent the control values plus or minus one standard deviation, i.e., the normal range. Each X represents one test animal.

Figure 8 shows a significant decrease in incorporation in the hepatic haem of biliary obstructed animals. The X's in Figure 9 show a high degree of variability but on statistical analysis (Student's T test) they do not differ significantly from the normal range.

2) Nephrectomy.

Figures 28 to 32 (page 36b) represent the percent of counts injected as 4-C^{14} delta-ALA which appeared in the bilirubin of anephric

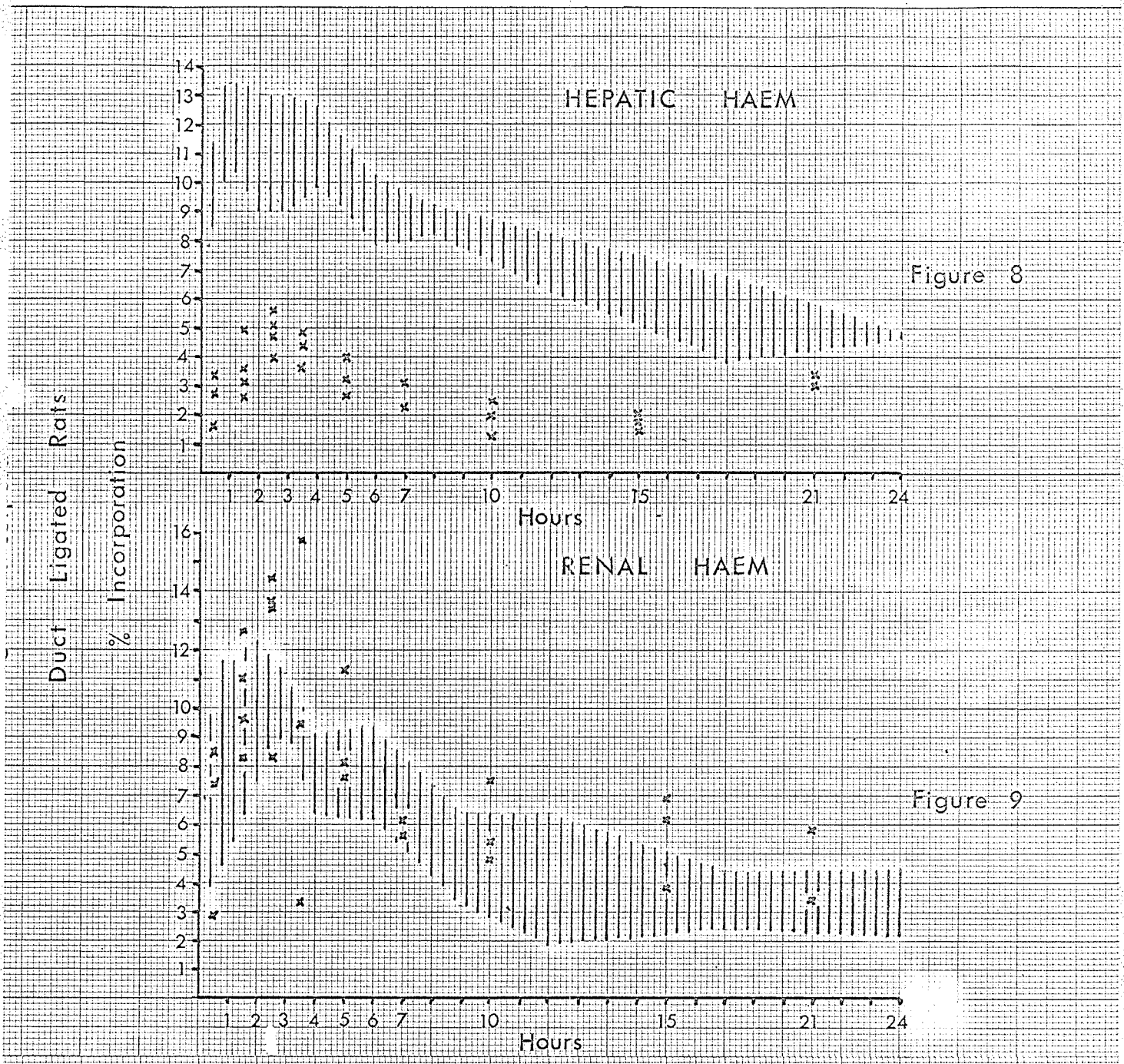
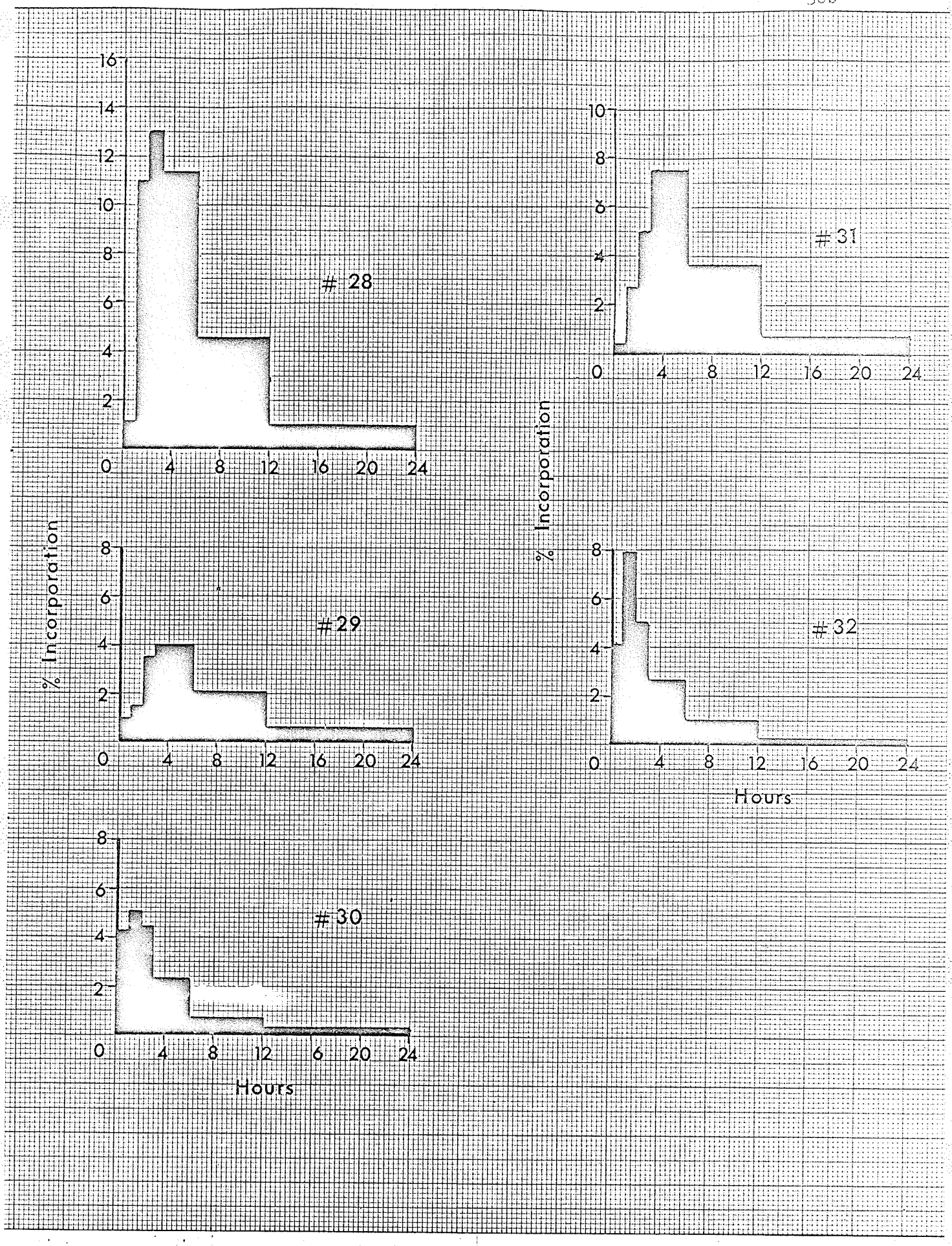


Figure 8

Figure 9


#28
#29
#30
#31
#32
% Incorporation
Hours
0
2
4
6
8
10
12
14
16
20
24

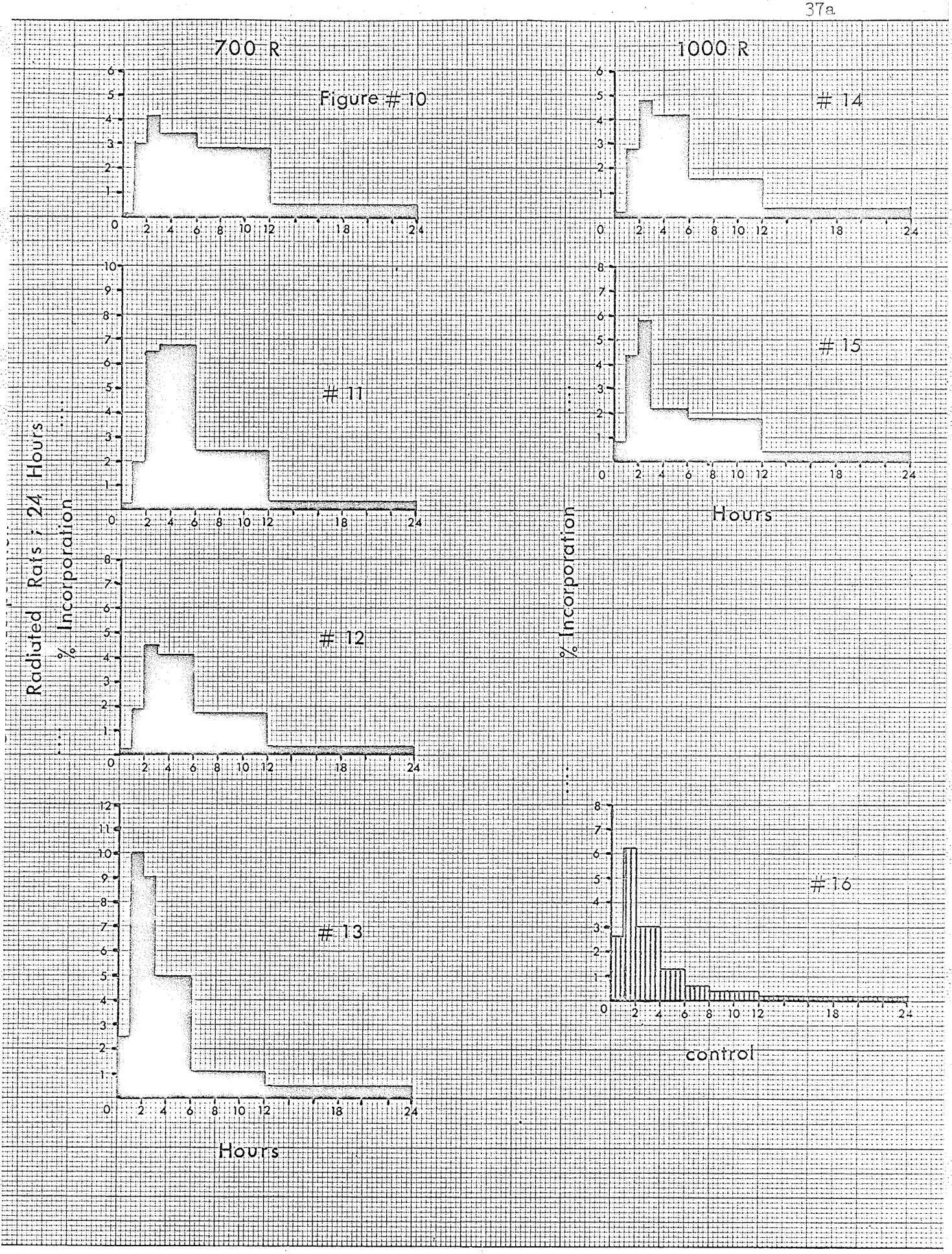
rats. As before the abscissa represents hours after injection and the ordinate, the percent of injected counts incorporated into bile bilirubin per hour.

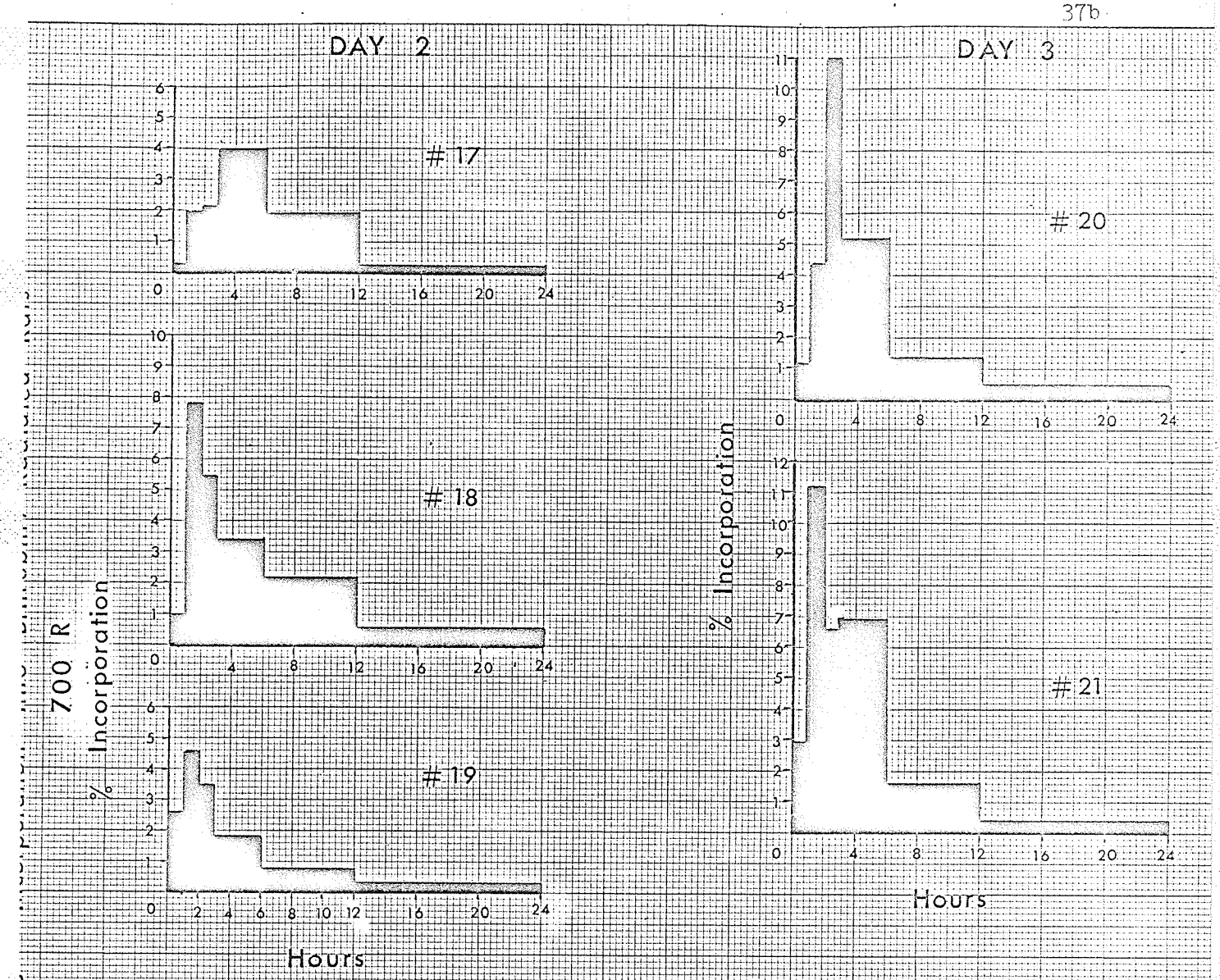
The results show a marked degree of variability in both pattern and total incorporation. Figures 31 and 28 have twenty-four hour incorporations of 60.38 percent and 98.5 percent, respectively. Figures 29, 30 and 32 incorporated 37.5 percent, 32.37 percent and 34.59 percent, respectively.

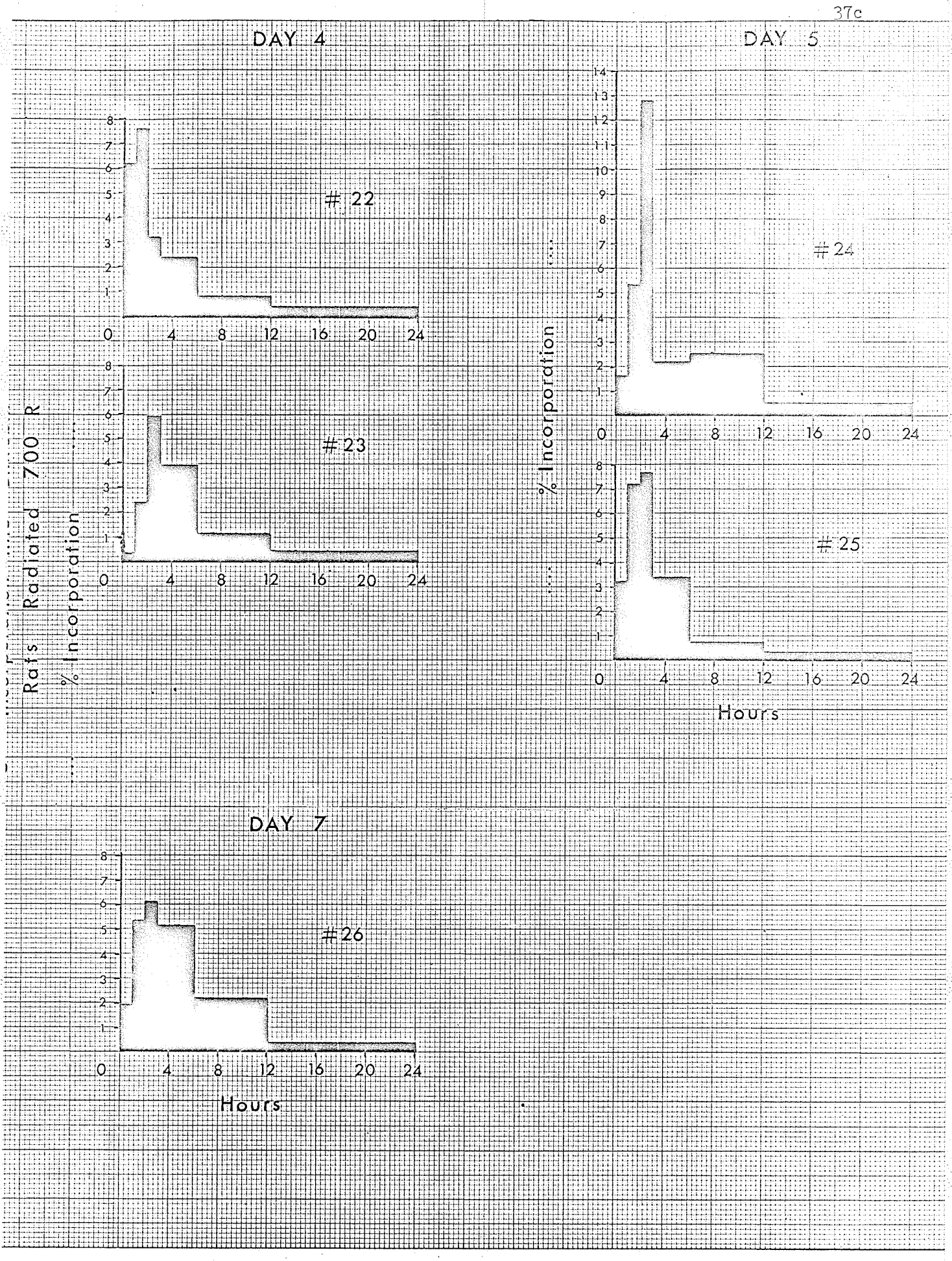
3) Radiation.

a) Bilirubin. Each of the bar graphs in Figures 10 - 26 (pages 37a-c) represents the bilirubin-isotope incorporation for a single rat with percent isotope incorporation represented on the ordinate and hours after injection on the abscissa. Figure 16 reproduces the control values of Chapter II. The radiation dosage and day of surgery and isotope injection are indicated for each group of animals: Figures 10 - 13, four rats, 700 R, operated and injected first day after radiation; Figures 14 and 15, two rats, 1000 R, first day; Figures 17 to 19, three rats, 700 R, second day; Figures 20 and 21, two rats, 700 R, third day; Figures 22 and 23, two rats, 700 R, fourth day; Figures 24 and 25, two rats, 700 R, fifth day; Figures 26, one rat, 700 R, seventh day.

An obvious characteristic of the graphs was the high degree of variability. Also seen in most of the animals was an apparent decrease in isotope incorporation during the first one or two hours after injection. This effect was clearly seen in ten of the fifteen rats and in five of the six rats injected on day 1 after radiation. The first hour's incorporation


700 R
1000 R
Figure # 10
14
11
15
12
16
13
Radiated Rats; 24 Hours
% Incorporation
Hours
control


DAY 2
DAY 3
#17
#18
#19
#20
#21
700 R
% Incorporation
% Incorporation
Hours
Hours


DAY 4
DAY 5
#22
#23
#24
#25
#26
Rafs Radiated 700 R
% Incorporation
Hours
0
4
8
12
16
20
24

in these animals was 0.75 ± 0.33 percent of the injected counts, while in control animals it was 2.68 ± 0.87 percent. This difference was significant to $P < 0.01$. The total twenty-four hour incorporation in these animals was also quite variable but averaged 39 percent, a moderate increase over the control mean.

b) Haem. Figure 27 (page 38a) depicts the percent of injected isotope incorporated into hepatic and renal haem of rats given 1000 R of radiation and injected with one microcurie of 4-C^{14} delta-ALA the first day after radiation. The ordinate represents percent of injected counts incorporated and the abscissa denotes the hours after injection. Values for liver haem are represented by X and kidney haem, by O.

In the animals studied, hepatic haem incorporation was unchanged while renal haem incorporation at hour one in two rats was 27 percent and 29 percent, almost triple the values in control animals. By the twelfth hour, there was no difference between control and radiated renal haems.

4) Amino-triazole (A.T.).

Figures 36 to 40 (page 38b) depict isotope incorporation in bile bilirubin in this group of animals. Ordinates and abscissae are as previously described and the arrows indicate the timing of the subcutaneous injection of 1 mgm/gm of 3-amino-1, 2, 4-triazole in each animal. Figure 41 represents a control animal injected with an equivalent volume of 0.9 percent saline. Although minimal increases in bilirubin-isotope content appeared following amino-triazole in every animal, the timing of

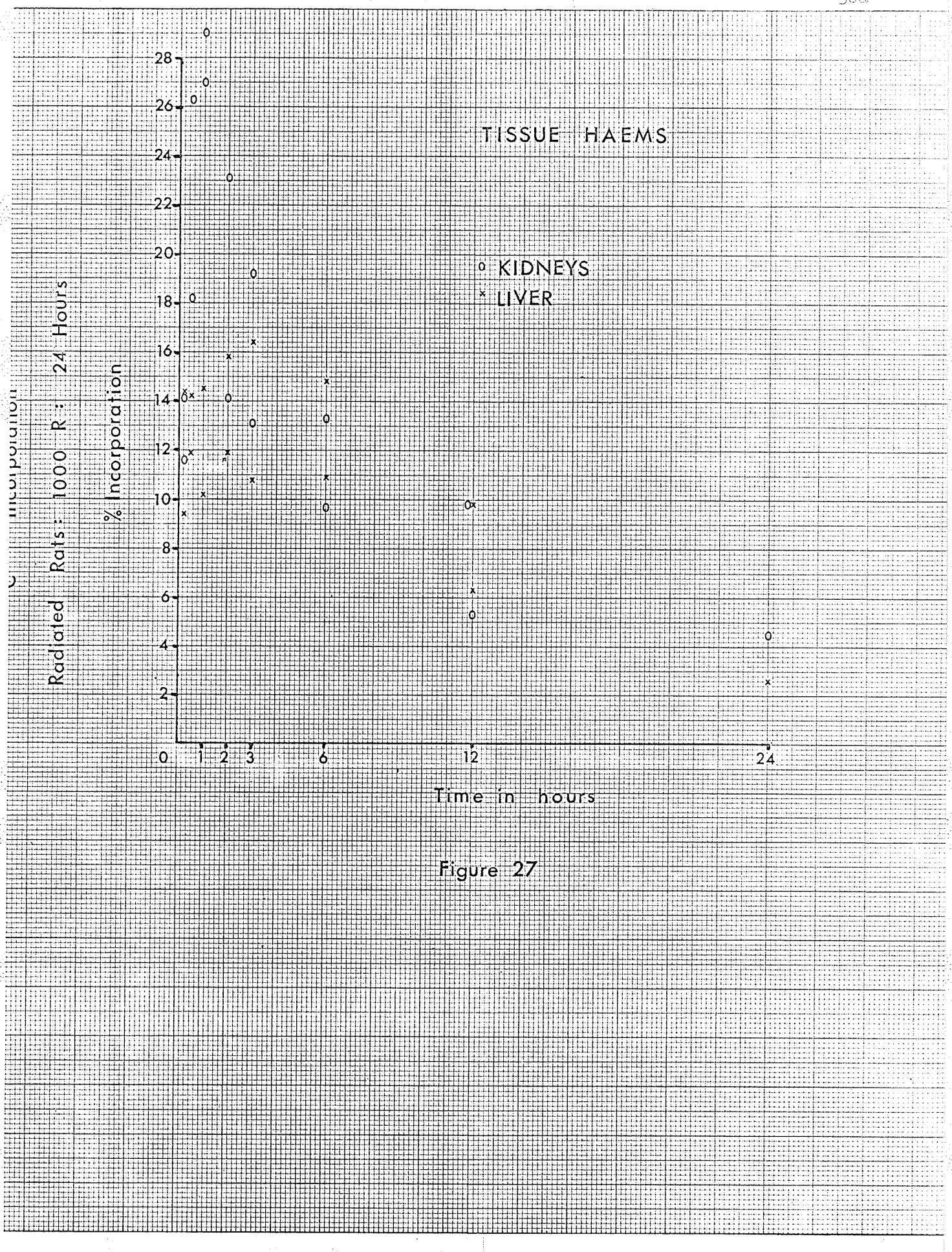
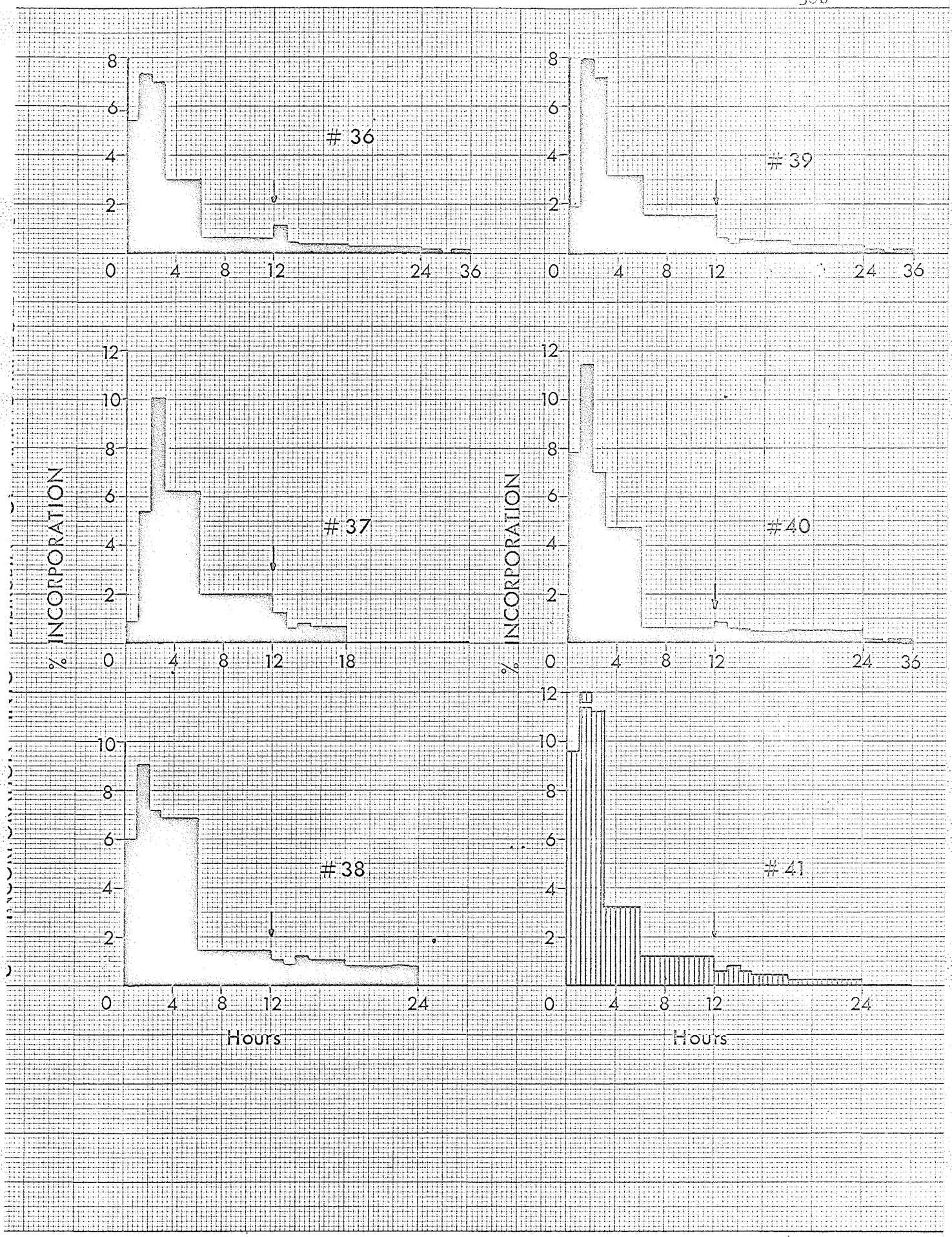

TISSUE HAEMS
KIDNEYS
LIVER
Radiated Rats: 1000 R : 24 Hours
% Incorporation
Time in hours
0
1
2
3
6
12
24
2
4
6
8
10
12
14
16
18
20
22
24
26
28

Figure 27


#36
#39
#37
#40
#38
#41
% INCORPORATION
% INCORPORATION
Hours
Hours

these increases was variable and a similar effect was observed in the control animal.

5) Phenobarbital.

a) Bilirubin. Bilirubin-isotope incorporation from injected 2-C^{14} glycine is shown in Figure 42 (page 39a). The ordinate represents the percent of injected radioactivity incorporated during a twenty-four hour period. The abscissa denotes phenobarbital dosage in milligrams per kilogram per day. Each X represents a rat which was fed throughout the experiment. The open squares represent rats starved from the fourth day of injection onward. Where no subscripts are shown the glycine dose was twenty microcuries. The subscript "100" indicates a glycine dosage of one hundred microcuries.

No significant increase in isotope incorporation was produced in animals receiving the "regular" 20 microcurie glycine dose at any level of phenobarbital. One animal in the 80 milligram phenobarbital group which received 100 microcuries of glycine showed a marked increase in incorporation (0.23 percent : control value of 0.08 percent) but this observation was not confirmed in a second experiment. There was no significant difference between "fed" and "starved" animals in the "regular" tracer dose group.

Several other animals received one microcurie of 4-C^{14} delta-ALA rather than glycine with the results illustrated in Table I (page 40).

PHENOBARBITAL INDUCTION OF CYTOCHROME P-450 VS SHUNT BILIRUBIN

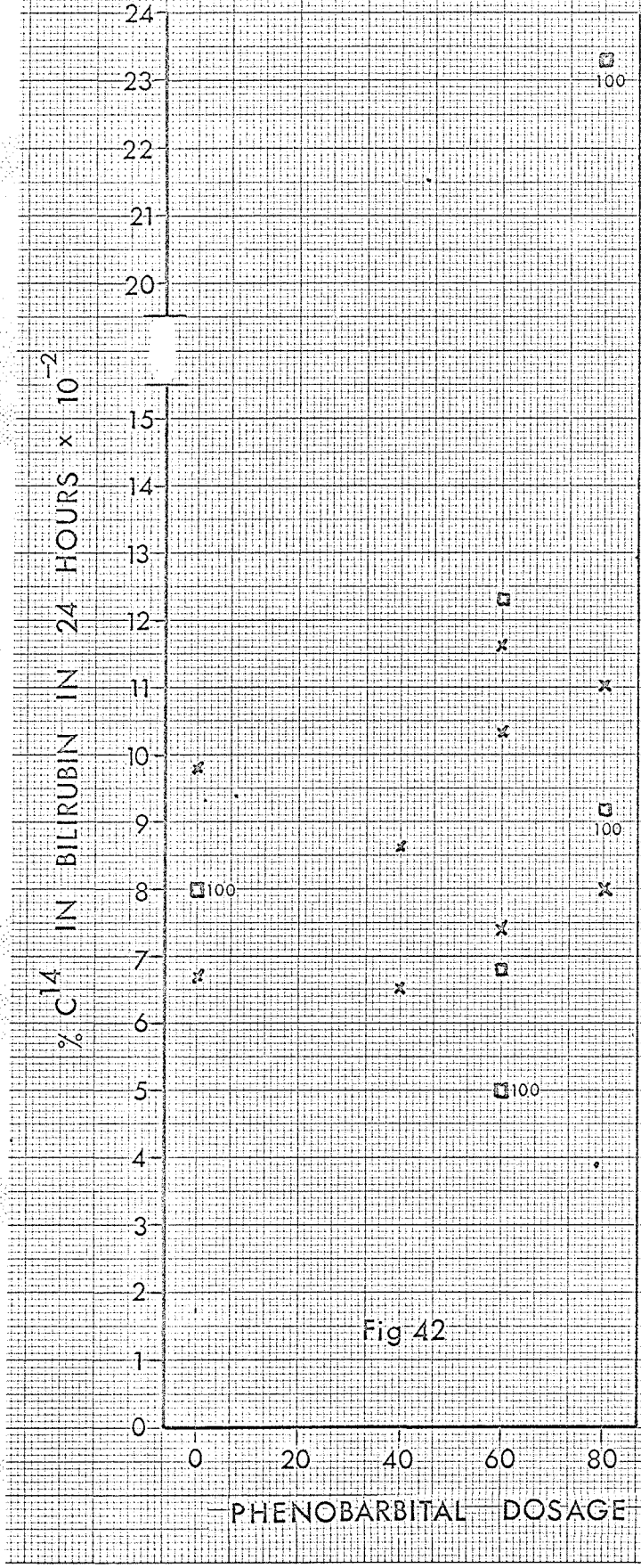


Fig 42

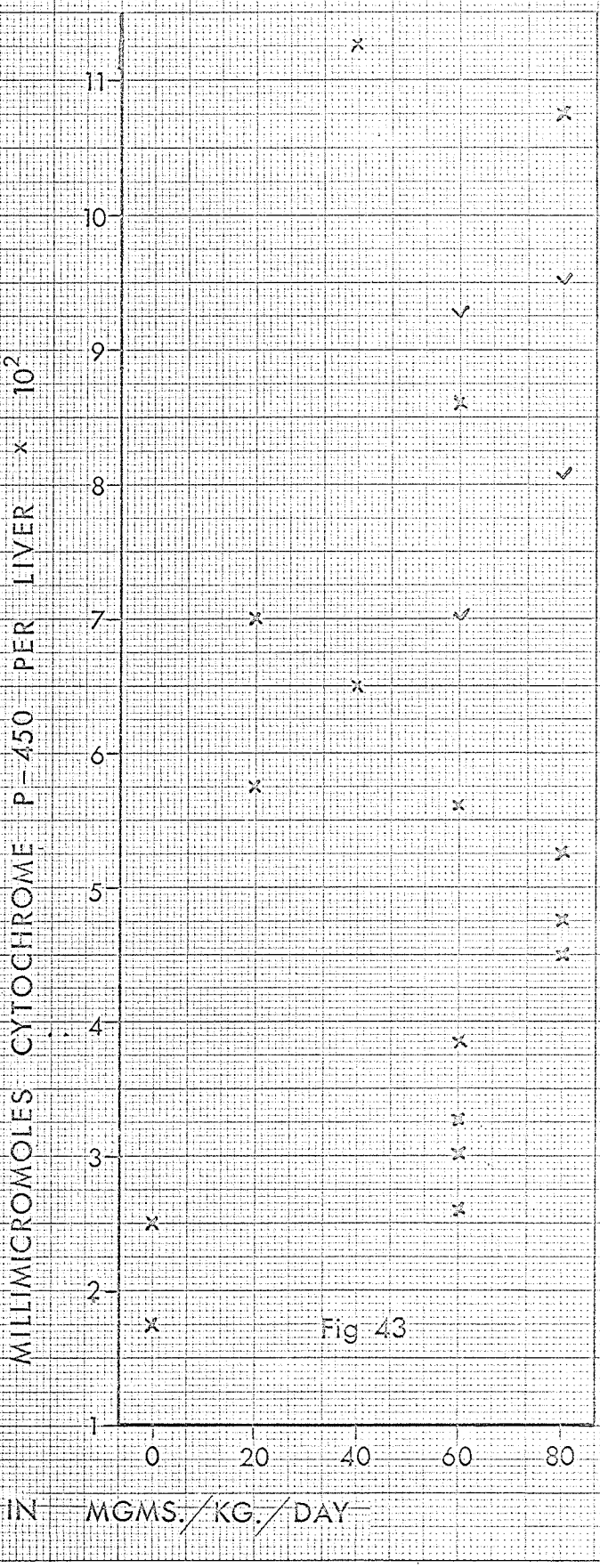


Fig 43

TABLE I

Dosage of Phenobarbital	Fed or Starved	24 hour Isotope Incorporation
0	fed	25.90%
60	fed	28.74%
60	fed	25.88%
60	starved	23.72%
80	fed	30.62%
80	starved	28.82%

No significant increase in isotope incorporation was induced even by the largest phenobarbital dose.

Table II (page 41) shows the twenty-four hour bile-bilirubin excretions for the animals described in Figure 42. Most animals excreted significantly more bilirubin than the control value of 1.2 to 1.3 milligrams per twenty-four hours (Chapter II). A considerable degree of variability was seen in these results.

b) Cytochrome P-450. Figure 43 (page 39a) depicts total hepatic cytochrome P-450 content as a function of phenobarbital dosage which is represented on the abscissa. The ordinate depicts millimicromoles of cytochrome P-450 per liver. X's represent fed rats and check marks, rats deprived of food from the fourth day of injection onward. A linear increase of three- to four-fold over control levels was produced at a dose level of forty milligrams. The effect produced at higher levels was variable and showed no further increases. Food deprivation had no consistent effect.

TABLE II

TWENTY-FOUR HOUR BILIRUBIN EXCRETION

Phenobarbital Dose	Tracer and Dose in Microcuries		Starved or Fed	24-hour Bilirubin Excretion
0	glycine	20	fed	1.96
0	glycine	20	fed	1.45
0	glycine	100	starved	3.32
0	delta-ALA	1	fed	1.50
40	glycine	20	fed	2.00
40	glycine	20	fed	1.49
60	glycine	20	fed	1.80
60	glycine	20	fed	2.32
60	glycine	20	fed	2.08
60	glycine	20	starved	1.93
60	glycine	20	starved	1.71
60	delta-ALA	1	fed	1.66
60	delta-ALA	1	fed	1.73
60	delta-ALA	1	starved	2.19
80	glycine	20	fed	2.59
80	glycine	20	fed	2.62
80	glycine	20	starved	1.97
80	glycine	20	starved	1.86
80	glycine	100	starved	2.67
80	glycine	100	starved	2.30
80	delta-ALA	1	fed	2.94
80	delta-ALA	1	starved	1.64

c) Haem. Isotope incorporation into hepatic haem in three rats which had received eighty milligrams per kilogram per day of phenobarbital for five days and were deprived of food from day four onwards was determined one hour after intravenous injection of one microcurie of 4-C^{14} delta-ALA. The observed values were: 16.65 percent, 16.44 percent and 12.85 percent, or a mean of 15.31 percent compared to the control mean of 11.9, a difference which was significant to $P < .05$.

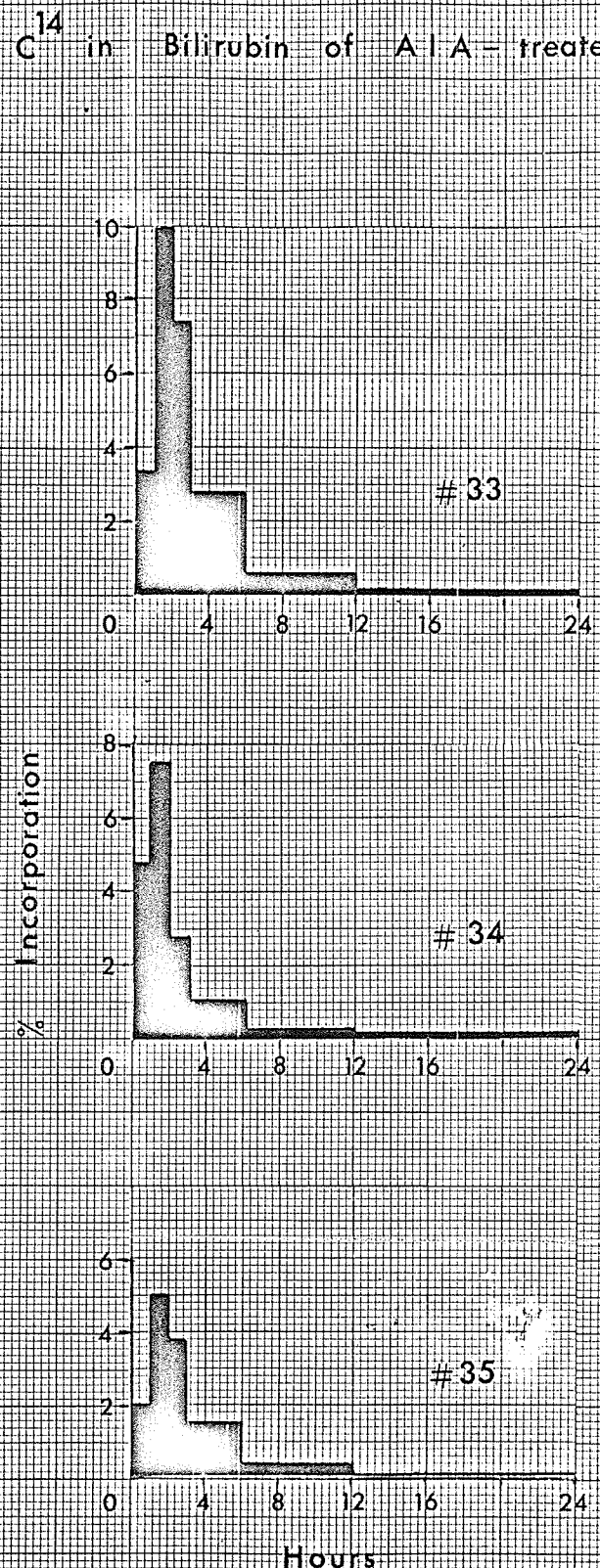
6) Allylisopropylacetamide (A.I.A.).

Mortality rate in the animals receiving A.I.A. was at least fifty percent prior to surgery. Symptoms such as weight loss of ten to twenty-five percent of total weight, lethargy, anorexia, constipation, and a partial flaccid paraplegia were commonly observed in treated animals. Only those animals whose bile fluoresced strongly in ultraviolet light were injected with 4-C^{14} delta-ALA. Figures 33 to 35 (page 42a) illustrate observations from three such animals.

The mean twenty-four hour bilirubin-isotope incorporation in these rats was 24.37 percent, not significantly different from controls. However, from hours twelve to twenty-four mean incorporation in controls was 0.35 percent per hour and in treated rats, 0.11 percent per hour--a difference which is significant to $P < .01$.

D. DISCUSSION

The effects observed in the biliary-obstructed group consisted of: 1) a marked increase in total bilirubin-isotope incorporation over


C^{14} in Bilirubin of AIA – treated Rats
% Incorporation
#33
#34
#35
0
2
4
6
8
10
12
16
24
Hours

the total twenty-four hour period although this was decreased over the first two hours; 2) a marked increase in bilirubin excretion during the first twelve hours after relief of obstruction; and 3) a marked decrease in hepatic haem-isotope incorporation.

The decreased incorporation seen in the first two hours may in part be due to dilution with the large pool of unlabelled bilirubin retained during the period of biliary obstruction. This pool appears to be cleared by the twelfth hour following relief of obstruction as evidenced by normal bilirubin excretion from twelve to twenty-four hours.

The decrease in hepatic haem incorporation accompanied by the increased bilirubin incorporation seems to present a paradoxical dissociation. Mechanisms which could account for this will now be considered.

A decrease in observed hepatic haem-isotope incorporation might be associated with: 1) decreased availability of isotope to haem-synthesizing sites; 2) decreased ability of haem-synthesizing sites to incorporate isotope, i.e., a dysfunction of haem synthesis; and 3) a small rapidly turning over pool of haems such that the isotope content observed at any point in time is low.

It is possible that decreased hepatic blood flow accompanying biliary obstruction might bring about a reduction of the availability of blood-borne isotope to haem-synthesizing sites (Szabo and Magyar, 1963). However, the haemodynamics of a liver in the first few hours following relief of obstruction are not well known and it is possible that a reactive hepatic hyperaemia may be in progress at this time bringing about

an actual increase in hepatic isotope delivery. This hypothesis is supported by the relatively normal glycine incorporations by hepatic protein observed in Chapter IV in similarly prepared rats. These possibilities must be considered in the light of the observed increase in bilirubin isotope incorporation and the concept that hepatic haems constitute a source of shunt bilirubin.

Biliary obstruction is known to be accompanied by such biochemical evidence of hepatic dysfunction as elevated SGOT and alkaline phosphatase, and eventually, disturbances in serum proteins (Zollinger, Britt, Ellison and Williams, 1963). The decreased hepatic catalase concentration referred to in Chapter I is consistent with the observed decrease in haem synthesis. A concurrent impairment of apoprotein synthesis is also possible in view of the general hepatic injury. The possibility of a small, very rapidly turning over pool of haemoproteins in a liver recovering from biliary obstruction is compatible with the observed increase in bilirubin-isotope content. However, we have evidence neither to confirm nor reject this possibility.

The increase in bilirubin-isotope content must arise either from hepatic or extrahepatic haem sources, or possibly, by an alternate pathway from haem precursors. Since the latter two possibilities have been discussed in Chapter I and are not directly related to the context of this chapter, further comment will be confined to the first possibility. If hepatic haems are indeed the source for the increased bilirubin labelling, there are two possible mechanisms: a rapidly turning over haemoprotein pool, or a rapidly turning over pool of free haem if apoprotein synthesis is disproportionately impaired. Both situations are compatible

with the observed decrease in hepatic haem-isotope content; hence a distinction cannot be made between them with this information.

The irradiated rats provided two significant findings: 1) a significant decrease in bilirubin-isotope incorporation during the first hour particularly evident in those observed soon after irradiation; and 2) a marked increase in the observed concentration of isotope in renal haems during the early hours after tracer injection.

Either an impairment in bilirubin synthesis or its excretion or an enhancement of bilirubin disposal by another pathway could account for the depression observed in the first hour. Since the quantity of bilirubin excreted is not decreased during the first hour, and isotope incorporation is not decreased after the first hour, a defect in the excretion of formed bilirubin is unlikely. This leaves the possibilities that either: 1) the formation of the source of a very early fraction of component I has been depressed; 2) the catabolism of only this fraction to bilirubin has been impaired; 3) an alternate pathway of bilirubin disposal may participate. A distinction between these possibilities cannot be made on the basis of the information at hand.

The great increase in renal haem isotope incorporation is related in time to the decrease in bilirubin labelling. Lemberg (1961) stated that a significant portion of renal haems are of the "a" rather than protoporphyrin type. It is not known whether the catabolite of these haems is bilirubin. If it is not, one would expect to observe an inverse relationship between the amount of isotope entering these haems and that entering bilirubin.

As to the nephrectomized rats, these animals underwent the most

traumatic surgical procedure and the twenty-four hour "recovery" period between surgery and injection was omitted. Omission of the "recovery" period was associated with a high degree of variability in delta-ALA incorporation into bilirubin. However, these studies did establish clearly that abundant shunt bilirubin is produced in the absence of renal haems.

Increased porphyrin fluorescence in bile and symptoms comparable to those seen in clinical human hepatic porphyria were observed in the A.I.A.-treated rats. The only consistent change in bilirubin-isotope incorporation was a decrease between the twelfth and twenty-fourth hours. One possible mechanism deserves mention with regard to this observation. It was stated in Chapter I that catalase has a half-life of about thirty hours. Schmid, Figen and Schwartz (1955) have shown that the hepatic content of this enzyme is depressed in "chemical porphyria". If catalase turnover contributed a significant portion of the shunt bilirubin fraction between the twelfth and twenty-fourth hours, this observation would be explained. The increased total hepatic haem synthesis in these animals (Labbe, Hanawa and Lottsfeld, 1961) in the presence of this shunt bilirubin pattern suggests an hepatic haem-bilirubin dissociation which is opposite to that seen in the "biliary obstructed" group.

As to the amino-triazole experiments, it must simply be concluded that under the conditions of our experiment the inactivation of catalase by A.T. had no consistent, observable effect on shunt bilirubin.

The observations in phenobarbital-treated rats have been partially analyzed under "Results". These animals showed: 1) a partially

dose-related increase in hepatic cytochrome P-450 content to three- to four-fold at a level of forty milligrams per kilogram per day; 2) no significant increase in bilirubin-isotope incorporation except in one rat given one hundred microcuries of glycine; and 3) variations in absolute bilirubin excretion, generally higher than control animals. These findings are against the hypothesis of Schmid (1966) that shunt bilirubin excretion is closely related to cytochrome P-450 turnover. It would appear that a direct relationship between induction of cytochrome P-450 and shunt bilirubin enhancement is produced only occasionally in the presence of a supra-tracer dose of glycine. The mechanism of such a phenomenon is uncertain but may be related to the observation of Granick (1965) that phenobarbital induces delta-ALA synthetase as well as cytochrome P-450.

CHAPTER IV

THE RELATIONSHIP BETWEEN HEPATIC PROTEIN, HAEMOPROTEIN, AND HAEM SYNTHESIS AND EARLY-LABELLED BILIRUBIN

A. INTRODUCTION

Thus far it has been established that: 1) the isolated perfused rat liver is capable of incorporating labelled delta-ALA or glycine into bile bilirubin (Robinson, Owen, Flock and Schmid, 1965); 2) rat liver mitochondria and cell sap incubated in 0.3 M sucrose are able to incorporate labelled glycine into bilirubin and carbon monoxide (White, Williams, Rother and Shafer, 1966). (Thus hepatic sources consisting of haemoprotein, haem and/or haem precursors are a source of shunt bilirubin.) 3) In biliary obstruction, labelling of shunt bilirubin is increased with reduced hepatic haem labelling; 4) A.I.A.-treated rats synthesize hepatic haem at an increased rate (Labbe, Hanawa and Lottsfeld, 1961) but show no increase in shunt bilirubin labelling (Chapter III). A similar relationship was documented in phenobarbital-treated rats in Chapter III; and 5) a marked increase in synthesis of a haemoprotein, cytochrome P-450 and a significant increase in hepatic haem synthesis could be produced with no significant change in shunt bilirubin. No clear cut haem-bilirubin relationship could be formulated from the above findings.

Haem, bilirubin and protein synthesis were studied in an *in vitro* rat liver system and the relationship between protein, haem and bilirubin synthesis was documented. Biliary obstruction caused the most

marked alteration in the shunt bilirubin-hepatic haem relationship observed in Chapter III. It was, therefore, of interest to determine the status of hepatic protein synthesis under these conditions. Further, injection of delta-ALA within three hours after establishment of bile fistulae in Chapter III was usually associated with a variable rise in bilirubin-isotope content. Determination of the bilirubin:haem:protein relationship under these conditions was, therefore, carried out. Finally, the relationship of shunt bilirubin, a haemoprotein (cytochrome P-450), total haem synthesis, and total protein synthesis was determined in rats given cycloheximide, a known inhibitor of protein synthesis (Ennis and Lubin, 1964).

B. MATERIALS AND METHODS

1) In vitro studies.

The livers of normal Sprague-Dawley rats killed by a blow on the head were immediately excised and perfused with sterile ice cold 0.9 percent saline to remove residual blood. They were then immediately homogenized in four volumes of 0.25 M sucrose with a Potter-Evehjem homogenizer using a teflon pestle. The homogenate contained very few intact cells and was maintained in an ice bath throughout the ensuing procedures. Prior to incubation one microcurie of 4-C^{14} delta-ALA was added to the homogenate which was then divided into eight aliquots of fifteen millilitres each. These were incubated in a shaker bath at 37°C for varying periods of time ranging from five to ninety minutes. After incubation each aliquot was removed from the shaker bath and quickly

frozen by immersion in methanol at -40°C. The frozen aliquots were then stored in a freezer pending haem and bilirubin extractions which were always carried out within one week. Aseptic technique was used throughout the above procedures.

At the time of extraction, each aliquot was thawed but kept in an ice bath. Each aliquot was subdivided into two equal fractions for the extraction of haem and bilirubin. Haem was extracted by the method of Labbe and Nishida (1957). To facilitate bilirubin extraction particulate material was removed from the homogenate. To accomplish this the bilirubin aliquots were treated with a Bronwell Biosonik sonicator for one minute each at which time no intact cells remained. They were then spun in a cold centrifuge at 10,000 rpm for ten minutes at 4°C. According to Bernstein, Ben Ezzer, Gartner and Arias (1966), 85 - 90 percent of the hepatic intracellular bilirubin is located in the soluble and microsomal fractions which were not sedimented by this treatment. Bilirubin extraction was, therefore, carried out from the supernatant. Carrier material was used in the extraction of both haem and bilirubin and the crystallized products were plated and counted. The specific activity of the haem and bilirubin was determined and the total amount of radioactivity in both haem and bilirubin in each aliquot was calculated by multiplying its specific activity by the amount of carrier which had been added. These calculations were based on the assumption that endogenous hepatic haem and bilirubin contents were very small compared to the amount of carrier added. The aliquot radioactivity content was then multiplied by the total number of aliquots to yield the total hepatic haem or bilirubin C^{14}

incorporation for each sample.

To study protein synthesis, ten microcuries of 2-C^{14} glycine was added to each 18 millilitre aliquot of an identical liver homogenate for incubation at 37°C. An aliquot was incubated for 180 minutes in a shaker bath and protein was then extracted. Ten volumes of 1.2 percent HCl in acetone were added to the homogenate and the globin protein extraction method of Kassenaar, Morrell and London (1957) was then used to extract and isolate protein. The powdered protein product was plated in 100 milligram quantities and radioactivity was counted in a Nuclear-Chicago Gas Flow Counter. A correction-factor curve for self absorbence was determined by counting planchets containing 1 to 200 milligrams of protein and this was applied to the calculations. Results were expressed as counts per minute per milligram of hepatic protein.

2) Acute surgical trauma and biliary obstruction.

a) Acute surgical trauma. The effects of acute surgical trauma on hepatic protein synthesis, hepatic haem, and shunt bilirubin synthesis were investigated by injecting rats intravenously with either one microcurie of 4-C^{14} delta-ALA (for haem and bilirubin incorporation) or 20 microcuries of 2-C^{14} glycine (for hepatic protein incorporation) within two to three hours after the establishment of bile fistulae. Bile collections were carried out in two rats and hepatic haem and protein determinations were carried out on the livers of two and four rats, respectively, excised 90 minutes after tracer injection.

b) Surgery plus recovery. Eight rats were permitted twenty-

four hours to "recover" in restraining cages following the establishment of bile fistulae. Five of these animals were then injected with 4-C^{14} delta-ALA (for bilirubin or hepatic haem studies) and three received 2-C^{14} glycine for protein determinations. Bile samples were again collected for twenty-four hours in blocks and the livers of four and three rats, respectively, were excised 90 minutes after the tracer injection for haem and protein extractions.

c) Biliary obstruction. The common bile duct was exposed and ligated in three rats as described in Chapter III. The abdominal incisions were then sutured and the animals were returned to their cages with free access to food and water. The bile ducts were recannulated forty-eight hours later and 2-C^{14} glycine was injected intravenously within three hours of surgery. Ninety minutes later the animals were killed by a blow on the head and their livers were excised for hepatic protein determinations. Cytochrome P-450 determinations were carried out in four similarly prepared rats with no tracer injections. Haem and bilirubin determinations in this system were described in Chapter III.

3) Inhibition of protein synthesis in vivo by cycloheximide.

All animals in this study received 1 milligram per kilogram of cycloheximide in an 0.9 percent saline solution at a concentration of 1 milligram per millilitre. In five rats bile fistulae were established twenty-four hours prior to cycloheximide administration. The rats were then given one microcurie of 4-C^{14} delta-ALA intravenously either 1 (3 rats) or 2-1/2 (2 rats) hours following cycloheximide and bile was collected in

blocks for twenty-four hours. Eight rats, similarly prepared and given cycloheximide twenty-four hours after establishment of fistulae, received either 2-C^{14} glycine or 4-C^{14} delta-ALA either 1 or 2-1/2 hours post cycloheximide to determine incorporation into protein and haem, respectively. These animals were sacrificed 90 minutes following glycine or delta-ALA injection and hepatic protein or haem was extracted. Isotope incorporation into the hepatic protein of three control animals not given cycloheximide was also determined 90 minutes after 2-C^{14} glycine injection.

Finally, the effect of cycloheximide specifically on the hepatic content of the haemoprotein cytochrome P-450 was determined. A series of rats were given 60 milligrams per kilogram per day of phenobarbital for five days to induce a high level of hepatic cytochrome P-450. On the sixth day each animal except one control was given 1 milligram per kilogram of cycloheximide subcutaneously. Starting at the time of cycloheximide injection and at hourly intervals thereafter to six hours, rats were given 20 microcuries of 2-C^{14} glycine intravenously. Each rat was killed by a blow on the head one hour after receiving glycine, and its liver was homogenized in 4 volumes of water, and divided into two equal aliquots for the determination of protein incorporation and cytochrome P-450 content. Hepatic cytochrome P-450 levels were determined in four control animals and three cycloheximide-treated animals who had not received phenobarbital to determine the sensitivity of the uninduced haemoprotein to cycloheximide.

C. RESULTS

1) In vitro studies.

The results of a typical experiment are seen in Figure 44 (page 54a).. The solid line depicts the percent of injected radioactivity incorporated into haem during the incubation. The broken line represents the incorporation of C^{14} into bilirubin in the same homogenate. The respective scales are described on the ordinate.

Radioactivity appeared rapidly and in almost parallel fashion in both fractions. The haem C^{14} content exceeded that of the bilirubin by a factor of at least ten in all aliquots. Peak incorporation for both fractions was attained at thirty minutes' incubation and this was followed by a plateau. That bilirubin incorporation values were not a contamination artefact was shown in a similar experiment using Fe^{59} as tracer, in which the bilirubin-haem incorporation ratio was one-hundredth of that observed using 4-C^{14} delta-ALA. An identical homogenate incubated with 2-C^{14} glycine yielded insignificant protein radioactivity of only 2.8 cpm/mgm at three hours' incubation time.

2) Acute surgical trauma and biliary obstruction.

a) Haem. Table III (page 55) lists hepatic protein, hepatic haem, and shunt bilirubin incorporation in these animals. "Control A" refers to rats with no bile fistulae. The value for hepatic haem in this group was obtained from Chapter II. "Control B" refers to four rats observed twenty-four hours after the establishment of bile fistulae.

Fig 44

Incorporation into the Haem and Bilirubin of Rat Liver Homogenate from 4 – C^{14} Delta – ALA

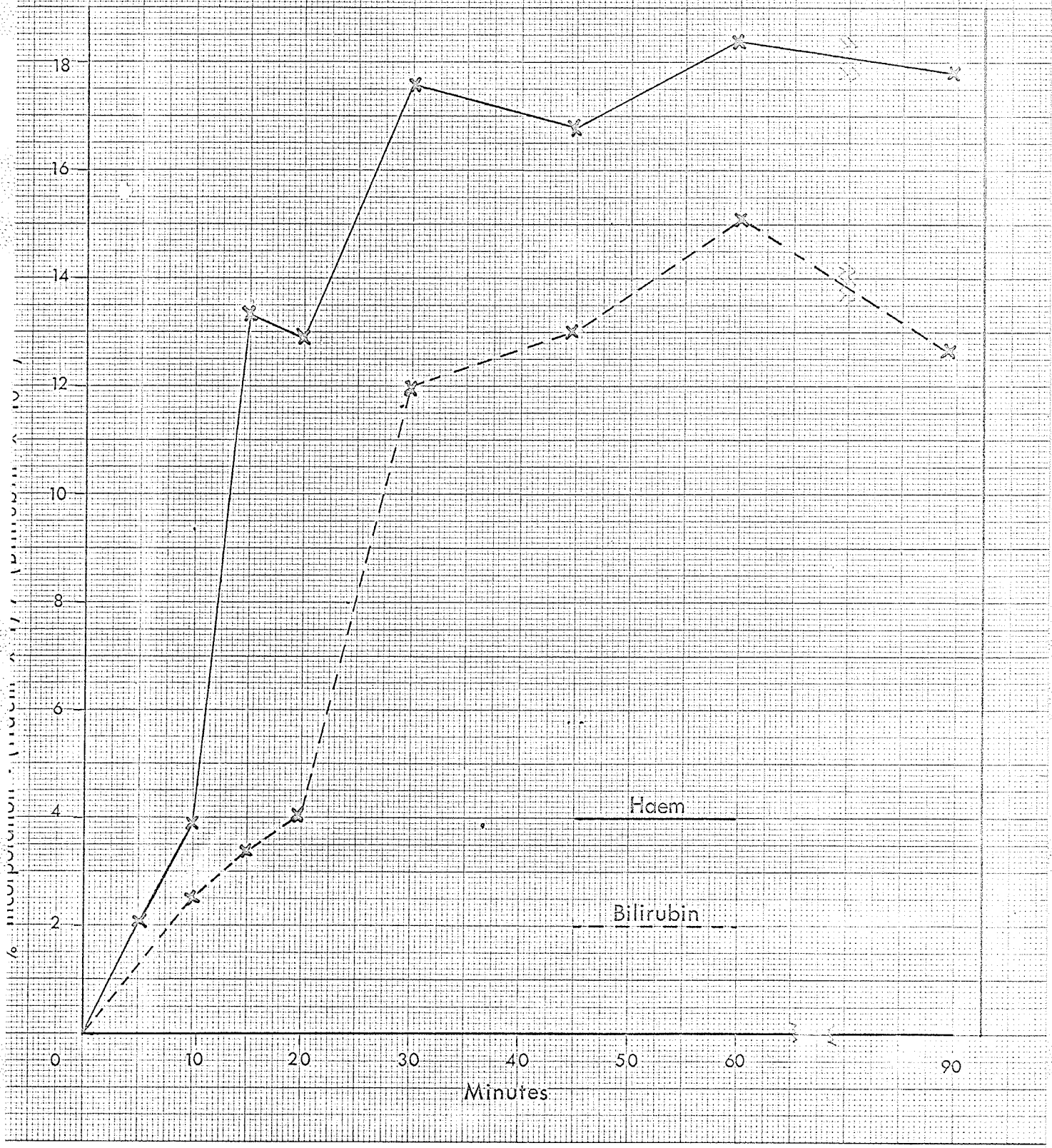


TABLE III

PARAMETER	CONTROLS		ACUTE SURGERY	BILIARY OCCLUSION	CYCLOHEXIMIDE	
	No Surgery "A"	24 hours Post Fistula "B"			24 hour Post Fistula 1 hour Interval	24 hour Post Fistula 2.5 hour Interval
Hepatic Protein cpm/mg 1.15 hour after 2-C^{14} glycine	88.4 84.8 mean=86.6	136.0 261.7 242.4 mean=213.4 (250% of "A")	58.3 68.9 75.9 100.7 mean=76.0 (88% of "A") (35% of "B")	111.5 170.3 145.1 mean=142.3 (165% of "A")	12.2 (6% of "B")	26.5 34.5 mean=31.5 (14% of "B")
Hepatic Haem % Incorporation 1.5 hours after 2-C^{14} delta-ALA	CH. II mean=11.5	10.8 10.4 9.0 8.9 mean= 9.8 (85% of "A")	9.6 8.4 mean= 9.0 (78% of "A") (92% of "B")	CH. II mean= 3.6 (31% of "A")	9.5 9.4 mean= 9.45 (96% of "B")	9.0 7.7 8.9 mean= 8.5 (87% of "B")
Bile Bilirubin % Incorporation in 24 hours after 4-C^{14} delta-ALA	--	28.8	38.4 31.3 mean=34.85 (121% of "B")	CH. II mean=52.11 (180% of "B")	33.16 34.31 32.92 mean=33.5 (116% of "B")	26.0 30.8 mean=28.4 (99% of "B")
Cytochrome P-450 millimicromoles per liver	mean (4) 305.8	--	--	mean (4) 154.4 (50.5% of "A")	167.8 (55% of "A")	mean (2) 178.4 (58% of "A")

The observations for hepatic haem in biliary obstruction have been obtained from Chapter III. Two rats injected with 4-C^{14} delta-ALA within three hours of establishment of bile fistulae had a mean haem incorporation of 78 percent of control "A" and 92 percent of control "B". Four animals injected twenty-four hours post surgery showed 85 percent of control "A" haem incorporation. There was overlap between the three groups and differences were not statistically significant. To facilitate comparisons, control "B" was hereafter adopted as the standard control system except where indicated.

b) Protein. Table III indicates that hepatic protein synthesis immediately following surgery was 88 percent of control "A" and 35 percent of control "B" in four rats. It was greatly increased in three rats (control "B") twenty-four hours after surgery to 250 percent of control "A". In three rats with biliary obstruction there was no impairment of protein synthesis with a mean value 165 percent of control "A" and considerable overlap with control "B".

c) Bilirubin. Bilirubin patterns in biliary obstruction were described in Chapter III. Table III shows that there was a slight increase to 121 percent in total twenty-four hour isotope incorporation into the bilirubin of two rats who received 4-C^{14} delta-ALA immediately after the establishment of bile fistulae. The pattern of isotope incorporation in these rats was moderately increased between hours 1 and 3. The pattern was not otherwise altered from normal.

d) Haemoprotein. Cytochrome P-450 levels in the obstructed rats were significantly reduced to 50.5 percent of control "A" as shown in Table III.

3) Inhibition of protein synthesis in vivo by cycloheximide.

a) Haem. Neither tracer interval resulted in significant inhibition of haem incorporation. The mean values were 96 percent in two rats injected one hour after cycloheximide and 87 percent in three rats injected 2-1/2 hours after cycloheximide. Results are expressed as percent of control "B".

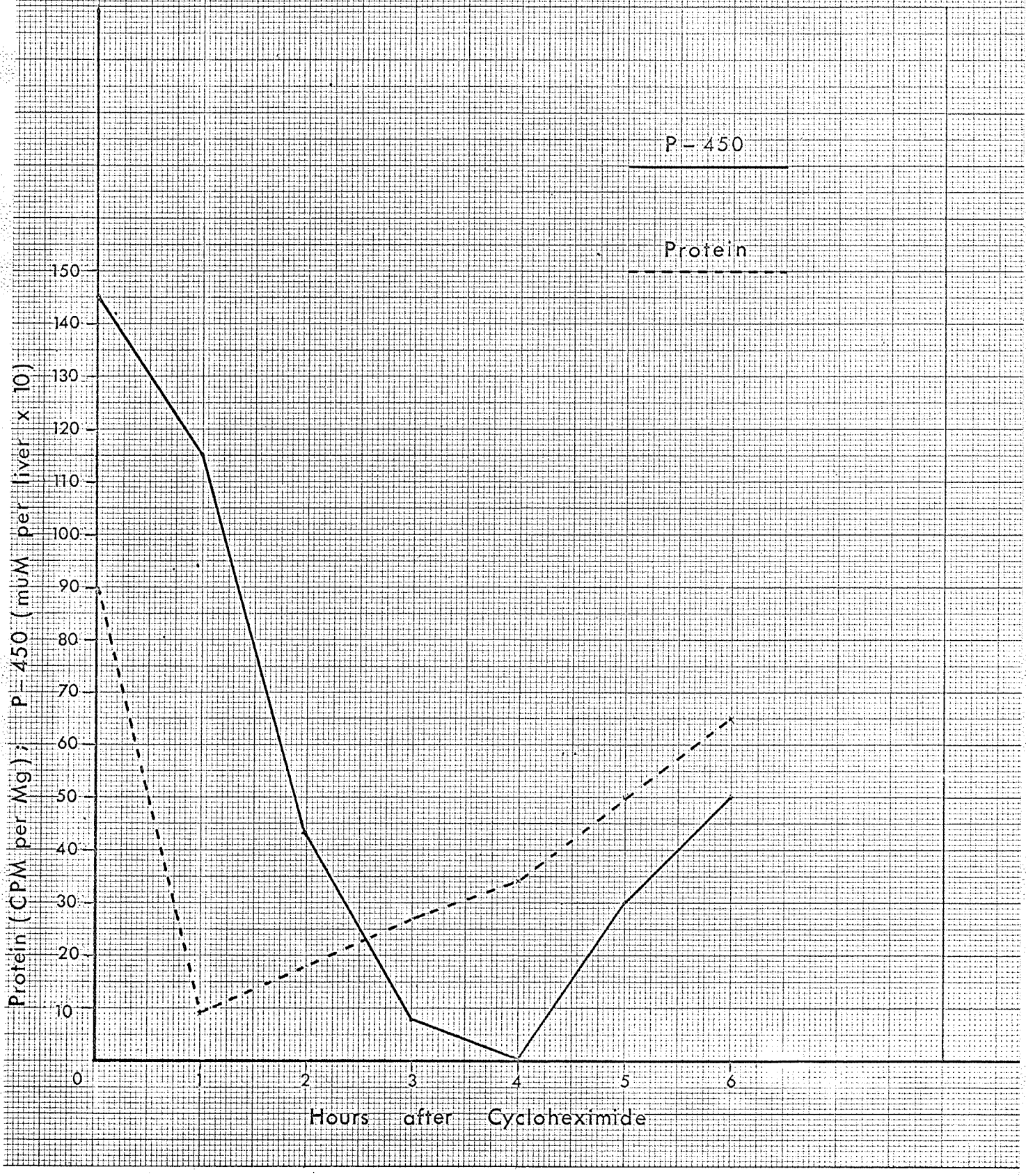
b) Hepatic protein. In two animals which had bile fistulae established twenty-four hours previously and received delta-ALA 2-1/2 hours after cycloheximide, protein incorporation was reduced to 14 percent of control "B". One rat received glycine only 1 hour after cycloheximide and showed the greatest degree of protein inhibition: 6 percent of control "B".

c) Bilirubin. No reduction in bilirubin incorporation was seen in animals which received delta-ALA either 1 or 2-1/2 hours after cycloheximide. The pattern of bilirubin incorporation in these rats did not differ significantly from normal.

d) Haemoprotein. Figure 45 (page 57a) shows hepatic cytochrome P-450 content (solid line) and hepatic protein 2-C^{14} glycine incorporation (broken line) in rats at various times after cycloheximide

C^{14} Incorporation into Hepatic Protein and Cytochrome P-450 Levels Following Cycloheximide

Fig 45



administration in phenobarbital-treated rats. Each point represents a single rat. Protein incorporation was lowest one hour post cycloheximide with a level of about 10 percent of control "A". Thereafter, it increased slowly to 70 percent of normal by six hours. As the rate of protein degradation exceeded that of protein synthesis, cytochrome P-450 content fell rapidly to its nadir at four hours and then began recovering. Table III indicates that the cytochrome P-450 of uninduced rats was also sensitive to cycloheximide.

D. DISCUSSION

The question of the relative contributions of hepatic "free haem" versus protein-associated haem in the evolution of early-labelled bilirubin led to the experiments described. Ideally such a problem would have been resolved by studying the relationship of early-labelled bilirubin to free haem and protein-bound haem under various conditions, thereby establishing the role of each haem form as a pigment source. Two factors which prevented such a definitive study were the heterogeneity of haemoprotein populations particularly with regard to half-life, and the lack of a method to obtain pure, non-artefactual tissue extracts of free haem and haemoprotein, respectively.

The first step was the demonstration (Figure 44) that tissue haem and shunt bilirubin synthesis proceeded in an in vitro system which was incapable of supporting protein synthesis. Under these circumstances, the possibility that newly synthesized haem became associated with previously formed apoprotein could not be ruled out; however, Figure 45 and Table III show that the level of the haemoprotein cytochrome P-450

fell simultaneously with general hepatic protein synthesis, at least *in vivo*. Synchronous haem and apoprotein synthesis were thus not prerequisite for normal component I shunt bilirubin synthesis. Nor was hepatic cytochrome P-450 content an important factor in determining shunt bilirubin production.

The results of the protein incorporation experiments were interpreted with cognizance of the non-specificity of the method as an indicator of haem apoprotein. Protein incorporation in the obstructed rats did not differ significantly from that in control group "B". There was no consistent correlation between "protein synthesis" and either haem or bilirubin incorporation when all the *in vivo* systems were compared. Comparison of the Table III parameters for the obstructed rats with those for control group "B" suggested an inverse relationship between bilirubin and haem incorporation; a relationship not noted in any other experimental group.

Variations in bilirubin incorporation in several groups of rats described in Chapter III which were injected within three hours of establishment of bile fistulae have been noted. Table III shows that the increase in bilirubin incorporation in rats observed the day of surgery was much less than that in the rats with obstructed bile ducts. Furthermore, protein-isotope incorporation was much less than in control group "B". Thus a threefold decrease in hepatic protein incorporation with no significant change in haem incorporation was seen in the presence of a minimal increase in bilirubin incorporation.

Finally, Table III shows haem and bilirubin synthesis in a system designed specifically to inhibit protein synthesis (i.e., in cycloheximide-treated rats). As in the acute surgery and obstructed groups no simple relationship between bilirubin and protein synthesis emerged.

Figure 45 and Table III illustrate that the level of the haemoprotein, cytochrome P-450 is directly related to protein synthesis. The constancy of component I bilirubin formation in the face of these gross changes in total protein synthesis and cytochrome P-450 content are strong evidence for the independence of its formation from these possible intermediaries. Further evidence is the dissociation of bilirubin-labelling and cytochrome P-450 content in biliary obstructed rats.

The possibility that cytochrome P-450 contributes to bilirubin in a minor way such that its elimination would not be detectable in overall component I kinetics was not ruled out by these results. In this regard, one may calculate the expected bilirubin yield of cytochrome P-450 turnover utilizing the values for: normal hepatic cytochrome P-450 content of 250 millimicromoles per liver (Figure 43), maximal enzyme half-life of 12 hours (Schmid, Marver and Hammaker, 1966), and the molecular weight of bilirubin of 584.65. Assuming no reutilization of the haem moiety, these values give rise to a bilirubin yield of 0.146 milligrams per 24 hours, or about 10 percent of the total daily bilirubin excretion, enough to account for almost the entire early-labelled pigment. Furthermore, if one considers the maximum possible yield in a phenobarbital-

treated animal (1500 millimicromoles per liver, Figure 45) and a half-life of two hours (Figure 45) the projected twenty-four hour bilirubin yield (assuming no reutilization) becomes 5.256 milligrams, much greater than the bilirubin excretion observed in any animal. It must be concluded, therefore, that the haem from this source is either largely reutilized or degraded through a different pathway.

The observed lack of effect of a reduction in hepatic cytochrome P-450 content on component I of early-labelled bilirubin suggests that degradation of even very rapidly turning-over haemoproteins may be relatively unimportant in the evolution of the component I peak. The impossibly high bilirubin yield calculated for the proposed direct degradation of cytochrome P-450 haem to bilirubin reinforces this thesis. The foregoing observations regarding cytochrome P-450 may apply equally to other short-lived haemoproteins.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Knowledge concerning the early-labelled bilirubin and its erythropoietic and non erythropoietic components has accrued since 1950. The pathogenesis of clinical hyperbilirubinaemias of both erythropoietic and hepatic origin has been linked with this fraction. More recently, interest in the non erythropoietic component of early bilirubin and its origins has mounted. The most likely sources which have emerged from recent studies were the rapidly turning over tissue haems of liver and kidney. The objective of the current investigation was to document the relationship between renal and hepatic tissue haem turnover and non erythropoietic shunt bilirubin and delineate more specifically the nature of the bilirubin precursors.

The relationship which emerged between the combined turnover of renal and hepatic haems and the synthesis of shunt bilirubin suggested that these sources could give rise to most or all of the non erythropoietic component. However, the findings that neither the absence of renal haem turnover in anephric rats, nor the greatly increased renal haem turnover in radiated rats was reflected in early bilirubin labelling minimized the importance of this organ as a source and directed attention toward the liver. The previous findings that the isolated perfused liver and liver mitochondrial suspensions could convert the simplest precursors to bilirubin strengthened the feeling that the liver was the primary source for the non erythropoietic component.

Subsequent studies on the relationship between hepatic haem turnover and shunt bilirubin suggested an inverse relationship between the two in biliary obstruction. In rats treated with A.I.A. or phenobarbital, substances previously shown to cause an increase in total haem synthesis, commensurate quantitative changes in bilirubin labelling were not observed. However, these observations were obviously inadequate as the basis to formulate a complete haem-bilirubin relationship.

The possible sources for component I shunt bilirubin in the liver include haemoproteins, free haem, and haem precursors. That component I may be further subdivided into at least two subcomponents with regard to its origin is implied by the differential effects of various agents on what appear to be a very early (0 - 3 hours) and a later (6 - 24 hours) phase of early bilirubin labelling. The effects of A.I.A. in blocking hepatic catalase synthesis and simultaneously reducing bilirubin labelling during the second phase imply that catalase and possibly other haemoproteins with similar half-lives may be important precursors of this subcomponent of the pigment. On the other hand, the lack of correlation between hepatic levels of cytochrome P-450 and tryptophane pyrrolase with very early bilirubin-labelling suggests that the very rapidly turning-over haemoproteins may be of lesser significance in the evolution of the very early subcomponent. This hypothesis is re-inforced by the observed lack of effect of cycloheximide on the very early subcomponent. These findings favour the implication of the non-protein sources, free haem and/or haem precursors, as being of major importance in very early bilirubin labelling. Although it appears likely that bilirubin production requires haem as a

substrate, the rapid conversion of Protoporphyrin IX to bilirubin has been demonstrated by Ibrahim, Schwartz and Watson (1966); however, labelled haem also appeared very rapidly suggesting that it might have a role as an intermediary in this experiment. Thus, from the present investigation, it appears likely that non erythropoietic shunt bilirubin arises primarily from hepatic sources. Of the two recently delineated subcomponents, the later appears to arise largely from the turnover of haemoproteins with half-lives similar to catalase; the very early subcomponent is probably largely derived from the rapid turnover of free haem or haem precursors.

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