

ONTOGENETIC CHANGES OF PROTEINS OF ENDOPLASMIC RETICULUM

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ABSTRACT

The proteins of the smooth and rough endoplasmic reticulum from fetal, immature, and adult male rats were compared after incorporation of two radioactively labeled precursors, ^{14}C -labeled amino acids and δ -aminolevulinic acid- ^3H by means of gel electrophoresis. The labeling patterns indicated that protein components present in two major electrophoretic bands underwent significant synthesis in fetal tissue while three actively incorporating protein bands were noted in adult tissue. Although the uptake of the amino acids- ^{14}C decreased for the smooth and rough elements of the endoplasmic reticulum as a whole during liver development, the qualitative patterns were not significantly different in adult and fetal livers. The over-all incorporation (disintegrations per minute per milligram protein) of the heme precursor into the smooth and rough elements also did not change with development. However, a change was noted in the distributional electrophoretic patterns with development. The estimation of molecular weight (by disc electrophoresis) and the incorporation of the heme precursor suggested the similarity of the two major protein bands to cytochrome P-450 and cytochrome b_5 , components of the endoplasmic reticulum, thought to be involved in the mixed-function oxidase system. The evidence indicated that in fetal liver, at a time when the oxidase capability was low, the amino acid incorporation into these two protein groups was the same as in the adult. The incorporation of the heme moiety, however, was different, decreasing in the cytochrome b_5 region and increasing in the cytochrome P-450 region during development. These results correlate with the increase in oxidase activity associated with liver development.

INTRODUCTION

A number of laboratories have reported investigations into the biogenesis of membranes and constitutive enzyme systems in developing mammalian liver (1, 13-18, 28). Such studies have shown that, at birth, enzymes generally concerned with homeostatic functions of the liver are low in activity. Raftell and Perlmann (15) have noted the presence of only several of the many adult-type antigens in the rat liver at 2 days after birth. Since an appreciable number were not seen, the appearance of enzymes is apparently initiated in some sort of

programmed manner after birth. The exact mode of synchrony is a function of the species studied (14, 15, 18, 25, 26, 28). The evidence would indicate the appearance of additional proteins to involve new synthesis and not simply the result of activation of preexisting material.

One of the principal components of the endoplasmic reticulum (ER) is the mixed-function oxidase system. The latter is markedly affected by exogenously administered or endogenously elaborated hormones and other substances. Further-

more, the response of the microsomal system to these agents is a function of the stage of tissue development. Thus, earlier work had indicated that drug metabolism in fetal and neonatal tissue is low but that it increases rapidly to adult levels very shortly after birth (19, 20, 21, 26, 37). Kato et al. (27) had reported that enzyme activity reached a maximum at 30 days, decreasing thereafter to about 50% of the adult activity in 150-day old rats. Further, the ability of this drug-metabolizing system to respond to the administration of a variety of "inducers," known for their potency in adult systems, was also low in fetal tissues and became more pronounced after birth (20, 22, 23). These results reflected some maternal control on the expression and induction of the enzymes (18, 22, 24). In contrast, some investigators (33, 34) have shown that aryl hydrocarbon hydroxylase, when induced with 1,2-benzanthracene, will show an increase in enzyme activity in fetal liver tissue, if the inducer is injected directly into the mother before birth.

The hemoproteins, cytochrome P-450 and cytochrome b_5 , have been shown by Dallner et al. (25) to occur in low concentration in 1 day old neonates as components of the mixed-function oxidase system. Indeed, these authors noted the difficulty in measuring the hemoproteins before this time. At 8 days after birth, however, the concentration of the latter reached 75-80% of the adult levels. It was felt that an understanding of the kinetics of synthesis of these hemoproteins in fetal tissues would be important in helping to elucidate the mechanisms involved in fetal development and their response to drugs and hormones.

In the present study, the microsomal proteins were studied by means of gel electrophoresis and by radioisotope methodology. Furthermore, the electrophoretic patterns and incorporation data were compared in fetal and adult livers. A difference has been found in the labeling of the heme components of fetal and adult liver proteins in the smooth and rough endoplasmic reticulum.

MATERIALS AND METHODS

Pregnant albino rats were obtained from Holtzman Rat Company, Madison, Wis.; the date of pregnancy was determined by sperm-positive smears. Fetal age was estimated from crown-rump measurements according to the procedure of Donaldson (7). Immature male rats, 70-90 g in body weight, were ob-

tained from Texas Inbred Company, Houston, Texas.

The animals were injected intraperitoneally with a mixture of 50 μ Ci of an amino acid- 14 C mixture (Schwarz reconstituted protein hydrolysate) and 100 μ Ci of δ -aminolevulinic acid- 3 H (New England Nuclear Corp., Boston, Mass., 2200 mCi/mmole). With the mothers anesthetized, the fetuses were exposed and each was injected intraperitoneally with a mixture of 2.5 μ Ci leucine- 14 C (New England Nuclear Corp., 312 mCi/mmole) and 10 μ Ci of the δ -aminolevulinic acid- 3 H (ALA). 30 min after injection of the isotopes, the animals were sacrificed, and the livers were removed, rinsed in cold 0.25 M sucrose, blotted, and weighed. The smooth endoplasmic (SER) and rough endoplasmic reticulum (RER) were isolated by a modification of the method of Dallner et al. (1) and Süß et al. (2), as described previously (3). The liver was homogenized in 5 vol of cold 0.25 M sucrose, centrifuged at 12,000 g for 10 min, and the precipitate was discarded. The supernatant was made 15 mM in cesium chloride, and a 3 ml sample was layered over a 2 ml cushion of 1.3 M sucrose (also made 15 mM in cesium chloride) for centrifugation in a SW50 rotor (Spinco Division of Beckman Instruments, Inc., Fullerton, Calif.) at 40,000 rpm for 3.5 hr. The fluffy layer at the interphase (SER) and the pellet (RER) were retained. The SER and RER fractions were then diluted with cold 0.25 M sucrose and centrifuged at 40,000 rpm in a Spinco 50 Ti rotor for 1 hr. The pellets were rinsed with sucrose and rehomogenized in 3 ml of the citrate-phosphate buffer of Süß et al. (2). After a 1 hr incubation at room temperature the samples were layered over 2 ml of cold 1.7 M sucrose for centrifugation in a 50 Ti rotor at 30,000 rpm for 16 hr. The membranes, at the interphase, were removed and diluted with 0.25 M sucrose and centrifuged at 40,000 rpm in 50 Ti rotor for 1 hr. The pellets from this step were rehomogenized in the solubilizing buffer

TABLE I
Protein Content of SER and RER of Liver from
Fetal and Adult Rats

	No. of determi- nations	Proteins (mg/g liver)		RER/ SER
		SER	RER	
Fetal	6	0.62 \pm 0.08	0.81 \pm 0.14	1.32
Adult*	18	3.26 \pm 0.25	4.85 \pm 0.33	1.49

* Data taken from reference 3.

The livers from 20-day fetuses were removed and the SER and RER components were isolated as described in the Methods section. The fractions are expressed as milligram of protein per gram wet weight of liver \pm the standard error of the mean.

of Hinman and Phillips (4) consisting of 0.01 M Tris-acetate buffer (pH 9.0), 1% sodium dodecyl sulfate (SDS), 0.001% ethylenediaminetetraacetate (EDTA), 2 M urea, and 0.1% β -mercaptoethanol, and electrophoresis was performed on the samples without any further treatment. Purity of the fractions was verified by electron microscopy.

Disc gel electrophoresis was performed in 5% acrylamide, 1% SDS, and 2 M urea, on portions of these fractions, as described by Hinman and Phillips (4). The electrophoresis buffer consisted of 0.1 M Tris-acetate (pH 9.0), with 1% SDS, and 0.01% EDTA. Two sets of gels were prepared for each each sample, with 600 μ g protein applied to each gel. The gels were subjected to electrophoresis at 2.5 ma per gel for 4 hr. One set was stained for 1 hr with a solution of 1% Buffalo Black, 7% acetic acid, and 40% ethanol, and destained by diffusion. The second set was sliced at approximately 1.0 mm intervals. The gel slices (1.5 mm thick) were prepared for counting by liquid scintillation techniques, using the methods described previously (3) which involved

one immersion of the gels in 0.5 ml of 30% hydrogen peroxide, and incubation at 50°C until the slices were solubilized (usually overnight). A 10 ml sample of scintillation fluid was added to each vial for counting. An estimation of the molecular weights of these ER proteins was made, utilizing the method suggested by Shapiro et al. (5), with thyroglobulin, catalase, bovine serum albumin, trypsin, and cytochrome *c* as standards for calibrating the disc gels.

Protein concentration was measured by the method of Lowry et al. (6), utilizing bovine serum albumin as the reference standard. A Packard Tri-Carb liquid scintillation spectrometer was used for the counting of all samples. Spectrometer settings for double isotope technology resulted in a 14% spillover of ^{14}C into the ^3H channel with no ^3H radioactivity in the ^{14}C channel. The counting efficiency for ^3H was found to be 20% while the efficiency for ^{14}C was 40%. Aquasol (New England Nuclear Corp.) was used as the scintillation fluid.

Cytochrome b_5 was isolated from adult and fetal liver by the method of Omura et al. (12).

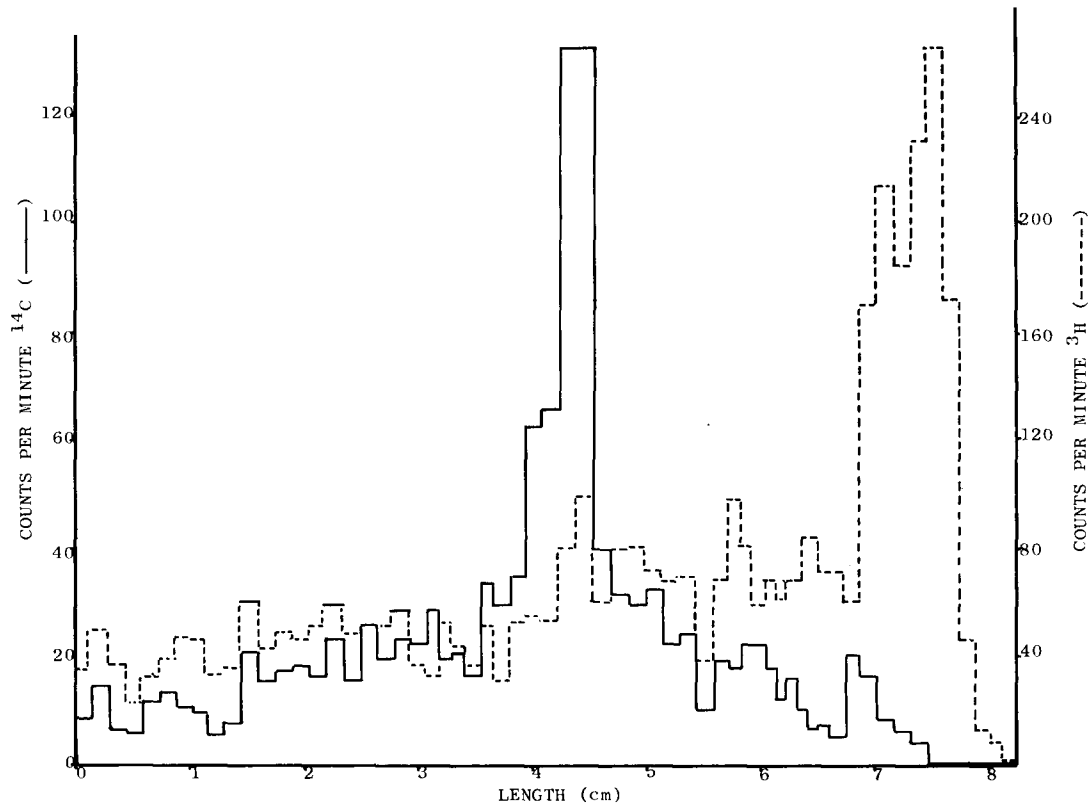


FIGURE 1 Profiles of radioactivity of protein components of SER from 20 day fetal liver. The ^{14}C -labeled amino acids and the ^3H -labeled δ -aminolevulinic acid were injected intraperitoneally 30 min before sacrifice. The SER and RER protein fractions were isolated as described in the Methods section and were analyzed by disc gel electrophoresis. The direction of electrophoresis was from left to right. The efficiency of counting was uniform across the electrophoretogram for both ^{14}C and ^3H .

RESULTS

The protein content of the SER and RER from fetal liver (Table I) is compared to the values obtained for suitably prepared fractions from adult liver (reported previously [3]). The RER protein

content is higher than that of the SER in fetal liver as seen with the immature and adult rats (3). The variation in the protein contents was greater for the fetal tissue than for adult liver, probably for the reason expressed by Dallner et al. (1), i.e., variable

TABLE II
Specific Activities of Fetal and Adult Liver Preparations

Age	No.	Specific activity (dpm/mg protein)					
		Amino acid- ¹⁴ C labeled			ALA- ³ H labeled		
		SER	RER	RER/SER	SER	RER	RER/SER
16 day fetus	2	11,700 (12,000; 11,300)	16,400 (13,700; 19,000)	1.41	47,000 (43,200; 50,800)	46,200 (43,700; 48,800)	0.99
20 day fetus	3	4,300 ±1,690	7,700 ±2,050	1.64	44,300 ±6,640	39,900 ±11,030	0.89
Adult	3	8,400 ±2,300	7,000 ±2,000	0.82	37,000 ±10,500	33,100 ±7,900	0.92

The animals were injected intraperitoneally with the isotope 30 min before sacrifice. The livers were removed and the SER and RER components were isolated as described in the Methods section. Values shown are given as disintegrations per minute per milligram protein ± standard error of the mean.

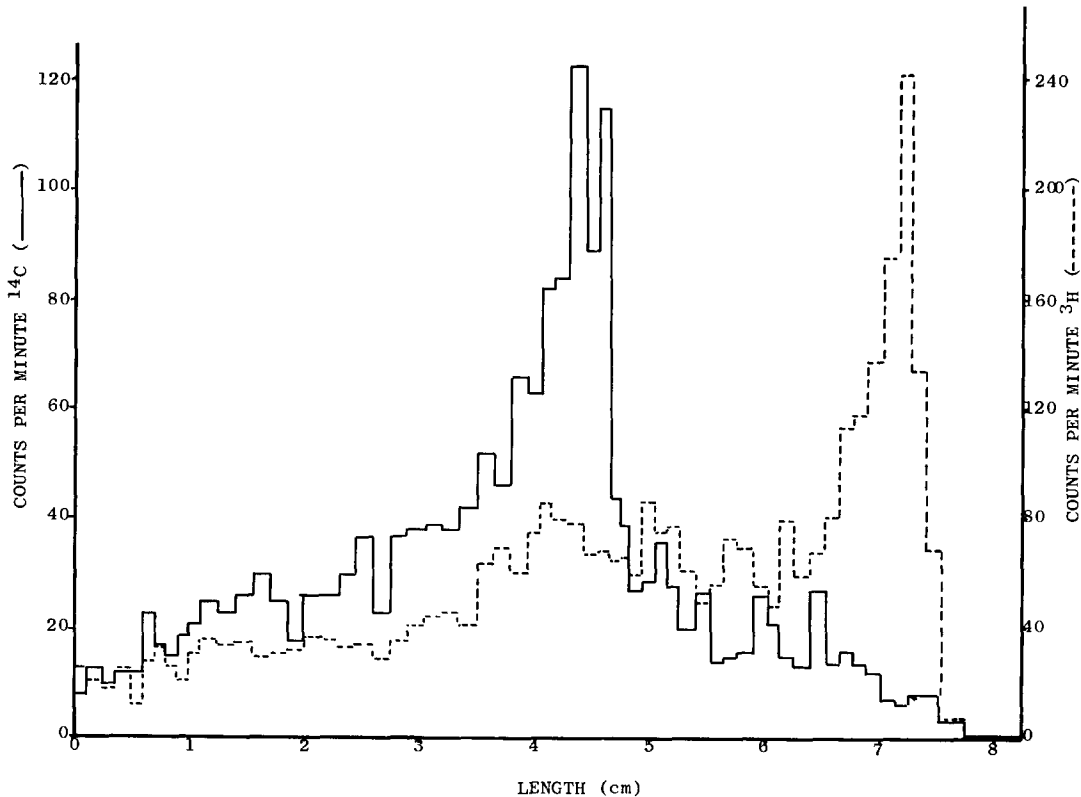


FIGURE 2 Profiles of radioactivity of protein components of fetal liver RER. See legend to Fig. 1.

amounts of glycogen present in fetal tissue. The values as reported here are in concert with the results presented by Dallner et al. (1).

The specific activities of the SER and RER proteins after a 30 min incorporation period with both amino acids- ^{14}C and ALA- ^3H are depicted in Table II. The rate of incorporation of amino acids into the SER and RER is higher in 16-day fetuses than in 20-day fetuses or adult animals. A higher amino acid incorporation in 18 day fetal liver was also reported in the work of Burraston and Pollak (17). The difference between fetal and adult tissue is reflected in the higher specific activity of the SER, and consequently in the lower RER/SER in the case of adult animals (Table II). These results are in keeping with the expected role of RER and SER in fetal tissue (1).

In contrast to the incorporation of labeled amino acids, the rate of ALA incorporation does not appear to change with development, nor does there appear to be any difference between the

SER and RER values, as reflected in the RER/SER ratio of approximately 1. Furthermore, the RER/SER ratio does not change after birth. A similar ratio of RER/SER was reported by Levin et al. (8) in immature male rats.

The profiles of radioactivity after incorporation of amino acid- ^{14}C and ALA- ^3H into the SER and RER proteins have been determined by disc gel electrophoresis; these data are depicted in Figs. 1-4. Little qualitative difference was apparent between the SER and RER disc gels with either label in fetal or adult liver preparations. Two areas of the disc gels obtained from fetal liver showed major incorporation; three areas appeared in the adult liver disc gels. These disc gels, typical of many experiments (at least five to six), show an obvious quantitative difference in the labeling pattern for both SER and RER proteins, after amino acid- ^{14}C and ALA- ^3H incorporation. The gel patterns from fetal liver (Figs. 1 and 2) showed that more amino acids- ^{14}C were incorporated into the

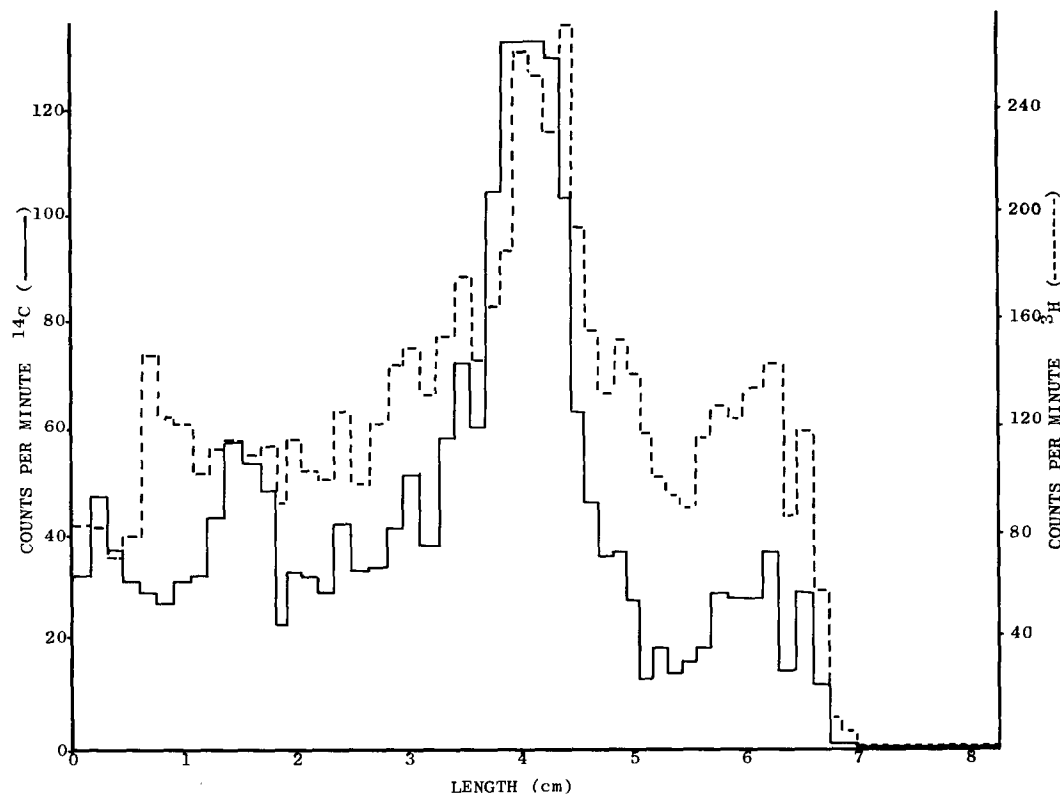


FIGURE 3 Profiles of radioactivity of protein components of immature liver SER. 50-70-g male rats were employed for the isolation of the liver SER. See legend to Fig. 1 and Methods section for details.

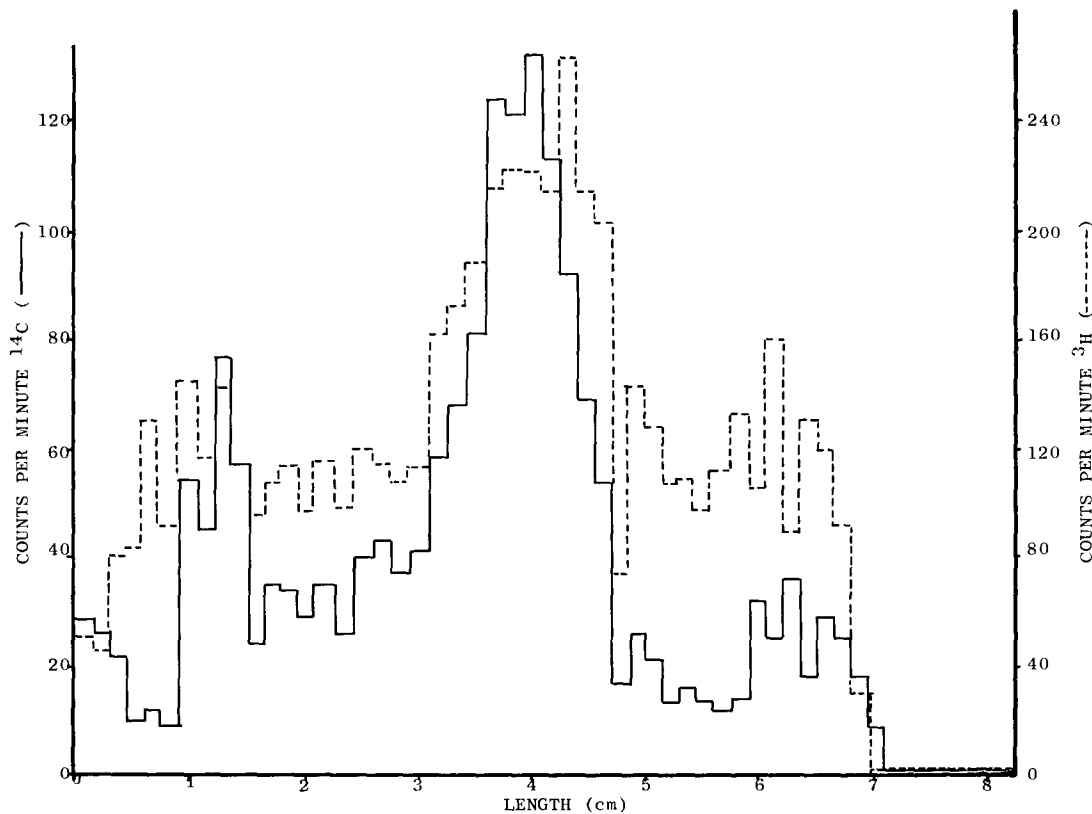


FIGURE 4 Profiles of radioactivity of protein components of immature liver RER. See the legends to Figs. 1 and 3.

TABLE III
Per Cent of Components in Fetal and Adult Preparations

Experiment	% of recovered radioactivity			
	SER		RER	
	³ H-label	¹⁴ C-label	³ H-label	¹⁴ C-label
Fetal liver				
3-5 cm	16-19	36-51	20-25	31-50
6-8 cm	32-39	4-11	24-35	4-14
Adult liver				
3-5 cm	34-43	48-54	26-44	33-55
6-8 cm	12	2-8	14-17	3-11

These values represent the range in per cent of the total heme radioactivity recovered in the specific regions of the disc gel from three experiments; where no range is given, all three values were identical. The 3-5- and 6-8-cm areas of the disc gels represent regions where cytochromes P-450 and *b*₅ would migrate.

3-5 cm area than ALA-³H; more ³H- than ¹⁴C-label was incorporated in the 6-8 cm area. The gel patterns from adult liver (Figs. 3 and 4), on the other hand, showed more ³H-incorporation into the 3-5 cm area than was seen in the 3-5 cm area of the fetal preparations. No explanation is available for the high background seen in the adult preparations. The fact that adult and fetal samples were handled alike would tend to indicate that the high background was not due to artifact of preparation. The ¹⁴C patterns in the 3-5- and 6-8-cm areas were comparatively similar in the fetal and adult liver fractions.

The differences, however, between the preparations were more obvious when the areas under the curves of these peaks were compared as shown in Table III. These results, presented as the per cent of the total radioactivity recovered in the gel slices, indicate no appreciable change in the amino acid-¹⁴C incorporation with liver development in either the 3-5 cm area or in the 6-8 cm

area, while a significant difference was apparent after ALA-³H labeling. Furthermore, the rate of incorporation of amino acids-¹⁴C into the 6-8 cm area was about one-fifth or less the rate of incorporation observed in the 3-5 cm area with both

fetal and adult preparations. The radioactivity of the 3-5 cm area labeled with ³H increased approximately twofold in the SER of adult liver, while the radioactivity of the 6-8 cm area was reduced threefold. In the RER, the increase in the

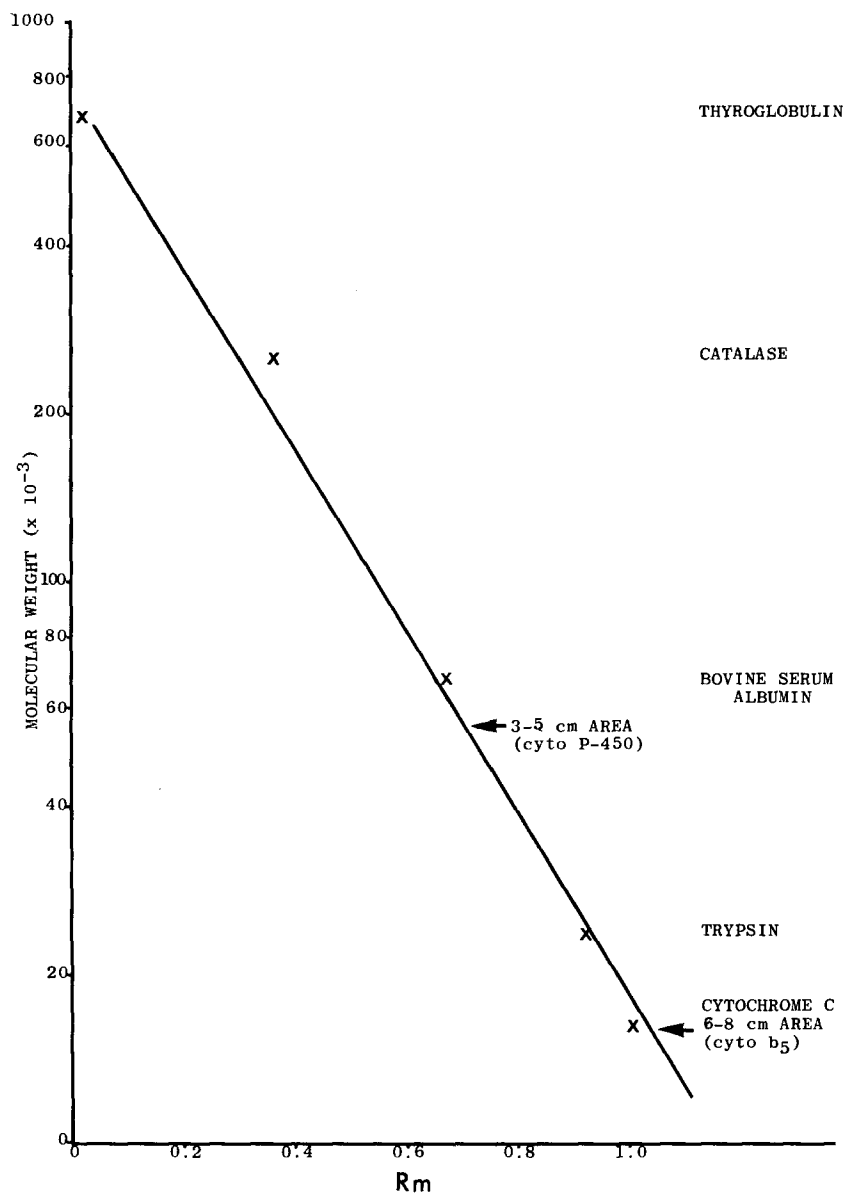


FIGURE 5 Molecular weight estimation of the major protein components of ER. Disc gels, which were prepared as described in the Methods section, were loaded with thyroglobulin, catalase, bovine serum albumin, trypsin, cytochrome *c*, and samples of the SER and RER from both fetal and adult liver preparations. The arrows represent the location of the major heme-labeled protein bands in relation to the standard proteins. R_m = migration of protein/migration of cytochrome *c*.

labeling of the 3–5 cm area was not as pronounced in adult liver; a reduction of approximately 50% was noted in the 6–8 cm region.

The molecular weights of the components migrating in the 3–5-cm and 6–8-cm ranges were estimated by SDS electrophoresis as shown in Fig 5 (with thyroglobulin, catalase, bovine serum albumin, trypsin, and cytochrome *c* used as standards). The molecular weight for the 3–5 cm material was calculated to be approximately 55,000; the 6–8 cm material, approximately 16,000. Estabrook et al. (9) have estimated the molecular weight of cytochrome P-450 as approximately 50,000, a value in concert with that of the 3–5 cm area. That the heme precursor is incorporated into this area is even more suggestive of this identification. Strittmatter et al. (10, 11) have estimated the molecular weights of cytochrome *b*₅ isolated from rabbit and calf livers as 16,900 and 12,700, respectively. Ito and Sato (35) and Spatz and Strittmatter (36) have more recently developed isolation techniques not requiring enzymatic digestion; molecular weights in the range of 16,700–25,000 were obtained. Thus, based on

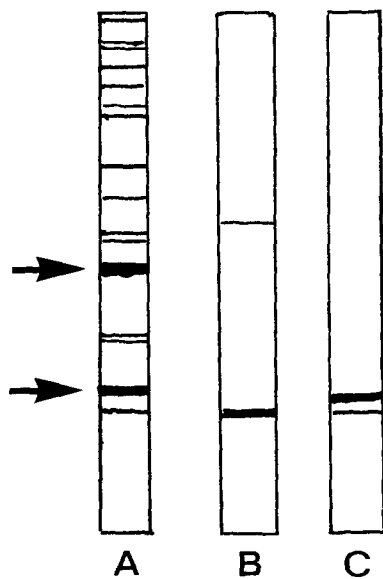


FIGURE 6 Disc gel electrophoretograms of proteins of SER compared to cytochrome *c* and cytochrome *b*₅. Disc gels comparing the proteins from a sample of SER (A) with standard of cytochrome *c* (B), and a sample of cytochrome *b*₅ (C) isolated by the method of Omura et al. (12). The direction of electrophoresis is from top to bottom. The two protein bands, shown by the arrows, are the major heme-labeled protein bands.

the molecular weight estimation and the incorporation of the heme precursor, the 6–8 cm area may be cytochrome *b*₅. Cytochrome *b*₅, isolated from rat liver, did indeed migrate to the same extent as the second ALA-labeled material, near that of the standard cytochrome *c*. This result is shown in Fig. 6.

DISCUSSION

The experiments reported here present several points of interest during fetal liver development. First, in fetal liver fractions, as was reported with adult preparations (3), two major proteins of the endoplasmic reticulum undergoing significant synthesis, i.e. incorporation of amino acids and ALA, are the hemoproteins, components of the mixed function oxidase system. This result is interesting in light of the evidence for the low activity of the latter microsomal system in fetal tissue as presented by several investigators (19–21, 26, 37). In this regard, it is important to note the difference in ALA-labeling patterns obtained with the SER of fetal and adult livers.

Hinman and Phillips (4) reported the presence, in salt- and detergent-washed rat liver SER and RER, of a single protein band with a molecular weight estimated at 50,000, which lent support for a single structural protein as the membrane element. Their results indicated that this protein represented approximately 30% of the total membrane proteins. Their protein fraction compares favorably with the major hemoprotein band found in our preparations. Additionally, Estabrook et al. (9) have noted that one single protein, cytochrome P-450, represented about 20% of the total protein of the endoplasmic reticulum.

A difference was noted in the incorporation of the heme precursor into hemoproteins, i.e. cytochrome P-450 and cytochrome *b*₅, of fetal liver, and adult liver SER and RER. The protein which appears to correspond to cytochrome P-450 incorporated labeled amino acids five times more effectively in both fetal and adult liver than that which corresponds to cytochrome *b*₅.

By utilizing labeled ALA as precursor, Greim et al. (38) reported that the half-life of cytochrome P-450, i.e. 22 hr, was only one-half that of cytochrome *b*₅, i.e., 45 hr. This faster rate of turnover of fetal liver P-450 may account for our results wherein a greater amount of radioactivity was present in the cytochrome *b*₅ than in the cytochrome P-450 region, particularly with regard to

the SER. In adult tissue, the heme radioactivity more nearly reflected the amino acid labeling data, suggesting a greater apo-cytochrome P-450 pool in fetal tissue than in adult tissue. The presence of apoproteins in adult animals has already been shown by several workers (8, 29-32) and, in fact, Baron and Tephly (30) have alluded to the possibility that the synthesis of heme in the adult may be the rate-limiting step in the production of cytochrome P-450.

This difference in the incorporation of the heme precursor into the fetal and adult liver ER fractions may be caused by a shift of the label from one hemoprotein to the other or from one precursor pool to another. This is seen as an equivalent decrease in the incorporation into the cytochrome b_5 -like protein with the increase in the labeling of cytochrome P-450-like protein after birth. In addition, the specific activities of the heme-labeled proteins of the SER and RER preparations did not change with development, which would appear to rule out any simple quantitative change in these components. A change of the relative levels of the cytochromes was observed by Dallner et al. (25) in fetal and adult tissues. In 1 day old neonates, cytochrome b_5 was in relatively greater concentration than was cytochrome P-450, while in 4 day old neonates the reverse was true. It is conceivable that liver development may be accompanied by an aggregation of apocytochrome components in the ER which is then followed by the generation of additional heme necessary for the formation of cytochrome P-450. This hypothesis would be in concert with the data reported in this manuscript.

We wish to acknowledge the valuable assistance of Dr. Mei Ying Yu, who prepared the cytochrome b_5 .

This work was supported by grants from the American Cancer Society, E-373, and Pharmaceutical Manufacturers Association.

Received for publication 2 August 1971, and in revised form 21 October 1971.

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