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MATURATION OF THE SULFOBROMOPHTHALEIN SODIUM–GLUTATHIONE CONJUGATING SYSTEM IN RAT LIVER *

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Sulfobromophthalein sodium, hereafter referred to as BSP, has frequently been employed to appraise hepatic function in the newborn infant (1–7). With but one exception (2), many studies have demonstrated delayed disappearance of BSP from blood and increased BSP retention in both the full-term and premature infant in the immediate postpartum period (1, 3–7). Subsequently, the rate of removal of BSP from blood gradually increased, and within a few weeks after birth, approached values found in older children and adults. In general, the impairment of BSP clearance in the first few days after delivery has been attributed to immaturity, and the gradual improvement to maturation of the factors involved in the hepatic removal of BSP from blood and the subsequent excretion of BSP into bile.

Based on a considerable body of evidence which has been accumulated within the past few years (8–15), it is now known that a major fraction of the BSP which is removed from blood and excreted into bile undergoes metabolic transformation within the liver. There appears to be general agreement that the major pathway of BSP metabolism in man, rat, dog, and other species involves conjugation with the tripeptide glutathione in thioether linkage (12–15). Recently, we identified an enzyme in the soluble supernatant fraction of liver that catalyzes BSP-glutathione conjugation (15). During conjugation, whether enzyme-catalyzed or not, bromine is released from BSP, and the sulfur group of glutathione attaches to BSP at the site of bromine removal (13, 15). Glutathione appears to be the only necessary substrate for BSP conjugation. No requirement for cofactor has been demonstrated (15).

The objective of the present study was to determine if the initial delay, then improvement in BSP clearance observed in the neonatal period, might be related to alterations in BSP metabolism. To achieve this, the BSP-glutathione conjugating system was appraised by assaying conjugating enzyme activity and measuring glutathione content of livers obtained from rats at various stages of development.

METHODS

BSP-glutathione conjugating enzyme activity and glutathione content were measured in livers of Sprague-Dawley rats of various ages. In assigning an age to fetuses *in utero,* it was assumed that the gestation period of the rat was 21 days from the date of fertilization. The animals were killed by a blow on the head, their throats cut, the livers removed rapidly, chilled, and weighed. Livers obtained from several litter mates of the younger rats were pooled in order to provide enough tissue for duplicate measurements.

1. Incubation procedure. Aliquots of liver were homogenized with various volumes of ice cold 0.1 M phosphate buffer, pH 7.8, either in a Dounce homogenizer (16), or in a motor-driven homogenizer utilizing a Teflon pestle.[1] The homogenates were then used for assay of BSP-glutathione conjugating enzyme. The homogenates were incubated in a test tube with BSP and glutathione in room air in a constant temperature water bath set at 37° C for 5 minutes. Incubation was terminated by addition of 0.36 ml of saturated ammonium sulfate and 5 ml of absolute ethanol (15). The contents of the tubes were thoroughly mixed, allowed to stand for 20 minutes at room temperature, then centrifuged at 2,500 rpm for 10 minutes.

2. Chromatography of BSP compounds. The above supernatant fluids were chromatographed in a descending system on Whatman no. 1 filter paper strips. BSP bands were identified by exposing the dried papers to ammonia vapors. In these studies, two bands were identified: one corresponded to BSP-glutathione, the other to

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[1] Purchased from Kontes Glass Company.

free BSP. The bands were cut out, placed in test tubes and eluted into distilled water for 30 minutes. A volume of 20 per cent KOH required to make the final concentration of KOH 2.5 per cent was then added to each tube, the contents thoroughly mixed, and the concentration of BSP in the eluate determined in a Beckman DU spectrophotometer set at 575 mμ. The chromatographic techniques and the methods of quantitating BSP on chromatograms have been described in detail in a previous publication (9).

3. Assay of BSP-glutathione conjugating enzyme activity. In preliminary studies, a 40 to 60 per cent ammonium sulfate fraction of rat liver supernatant suspended in 0.1 M phosphate buffer, pH 7.8 (supernatant fraction remaining after centrifuging liver homogenate prepared in 0.1 M phosphate buffer, pH 7.8, at 144,000 G for 30 minutes in a Spinco ultracentrifuge, model L), was used as the enzyme preparation to determine optimal quantities of BSP and glutathione required for enzyme assay. One ml of enzyme was mixed in a test tube with varying quantities of glutathione in 0.1 ml and BSP in 0.2 ml of 0.1 M phosphate buffer, pH 7.8, and incubated for 5 minutes in a constant temperature water bath set at 37° C. The quantity of BSP conjugated in each tube was then determined. Maximal BSP conjugation was observed when the incubation mixture contained 0.5 to 1.0 mg BSP and 2 to 6 mg glutathione. Addition of larger quantities of BSP, 1.5 to 4 mg, or of glutathione, 8 to 12 mg, resulted in depression of maximal BSP conjugation.

The quantities of substrate found to be optimal with the ammonium sulfate fraction of enzyme were then tested with liver homogenates. One ml of liver homogenate containing varying amounts of liver was incubated with 0.75 mg BSP contained in 0.2 ml and 4 mg glutathione in 0.1 ml phosphate buffer for 5 minutes at 37° C. Two types of blanks were used in the initial studies. In the first blank, 1 ml of liver homogenate was mixed with glutathione, saturated ammonium sulfate, and absolute ethanol; then BSP was added. In the second, 1 ml of phosphate buffer was substituted for liver homogenate. BSP and glutathione were added and the mixture incubated for 5 minutes at 37° C before termination of incubation with ammonium sulfate and absolute ethanol. Although chromatograms of the blanks did not disclose a BSP conjugate band, areas corresponding to this band were cut out from the chromatograms, eluted, and the absorbance substracted from the absorbance of the eluted BSP conjugate to yield a net absorbance due to BSP conjugate. The two blank readings were virtually identical. Therefore, in most of the studies, only the first type of blank was used. The amount of BSP conjugated by tissue obtained from a single liver was found to be proportional to the quantity of liver present in the incubation mixture, and thus presumably to enzyme concentration when up to 0.2 mg BSP was conjugated in 5 minutes (15). Therefore, this system appeared to be satisfactory for assay of enzyme activity in liver. In the present studies 40 to 250 mg of liver was contained in the 1 ml of liver homogenate. The larger amounts were used when livers of the very young animals were assayed for enzyme activity, since virtually no enzyme activity was detected with the smaller aliquots of liver. To compare enzyme activities of different livers, the results have been reported in terms of two reference standards. Most of the data is expressed as milligrams of BSP conjugated per gram of liver wet weight per 5 minutes. In some studies enzyme activity is also expressed per milligram of protein nitrogen. Protein nitrogen was determined by micro-Kjeldahl analysis on samples of liver homogenate digested before and after precipitation of protein with 10 per cent trichloroacetic acid.

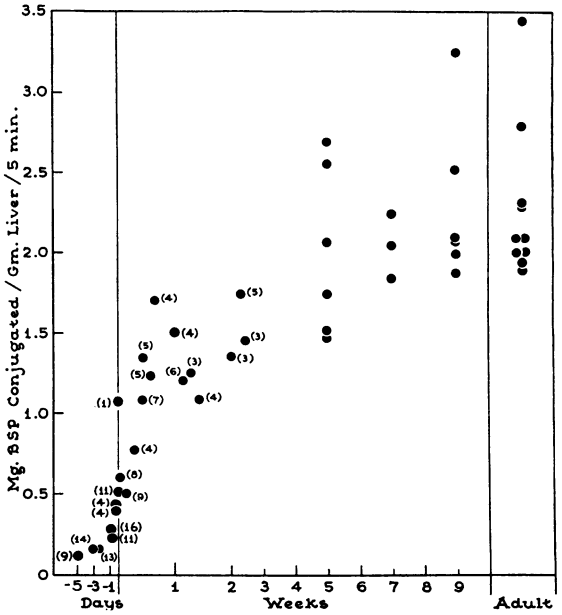

FIG. 1. RELATIONSHIP OF BSP-GLUTATHIONE CONJUGATING ENZYME ACTIVITY IN RAT LIVER TO AGE. Enzyme activity is expressed as milligrams of BSP conjugated per gram of liver wet weight per 5 minutes. Each point represents the value for either a pool of livers obtained from litter mates or for a single animal. The number of animals in each pool is listed in parentheses adjacent to the pertinent point. Male and female rats were included in the pools up to 3 weeks post partum. Rats 5 weeks of age and older were males. The vertical line at the left of the figure represents the date of delivery. For fetuses obtained *in utero,* this line is equivalent to day 21 of the gestation period, or the expected date of delivery.

4. Determination of liver glutathione content. Duplicate slices of liver, weighing approximately 400 to 500 mg each, were assayed for glutathione content by the method of Grunert and Phillips (17). The results were averaged and expressed as milligrams of glutathione per gram of liver wet weight.

RESULTS

1. BSP-glutathione conjugating enzyme activity. The data obtained from rats *in utero* through

TABLE I

Concentration of protein nitrogen in liver of rats at different ages

Age*	Protein nitrogen
days	*mg/g liver wet wt*
−4	18.5
−3	19.2
−2	19.3
−2	18.6
−1	18.4
−1	16.1
+9 (hours)	26.0
+1	26.8
+1	23.1
+2	16.3
+2.5	22.8
+3	19.0
+4	23.3
+5	24.1
+5	16.0
+7	28.8
+9	26.2
+10	30.1
+80 to 90	27.2
	29.9
	25.2

* Zero taken as day of delivery.

adult life are summarized in Figures 1 and 2. It is apparent that enzyme activity expressed in terms of grams of liver wet weight is very low in fetal liver prior to delivery, approximating only 5 to 20 per cent of adult values during days 16 to 21 of the expected 21-day gestation period. After delivery a rapid increase ensues, and by the third postpartum day liver enzyme activity is approximately 50 per cent of adult values. Subsequently, a more gradual rise in enzyme activity is observed, with adult levels reached by about the seventh postpartum week. At this age, rats weighed approximately 135 g, whereas the adult animals averaged 370 g. A similar rapid increase in enzyme activity seen after delivery is apparent also when values are expressed in terms of protein nitrogen (Figure 2). Although protein concentration in liver increases after delivery (Table I), it does so to a lesser extent than the rise in enzyme activity.

Conjugating enzyme activity was also measured in maternal liver obtained at various stages before and after delivery and compared with values obtained in adult nonpregnant female rats (Figures 3 and 4). Two separate series of animals were examined at an interval of approximately 1 year. The values for enzyme activity in adult control females were somewhat higher in the second group than in the first group of animals. Within each series of rats, enzyme activity was moderately depressed during the last 5 days of the gestation period, then returned to normal in the early postpartum period, with the suggestion of an additional rebound above the normal range.

The effect of mixing fetal and adult liver homogenates on conjugating enzyme activity was determined in order to detect the possible presence of an enzyme inhibitor in fetal liver or an enzyme activator in adult liver. The resultant enzyme activity of such mixtures was found to be equal to the arithmetic mean of the activities of the original homogenates (Table II).

2. Liver glutathione. The data are summarized in Figure 5. Liver glutathione concentration in

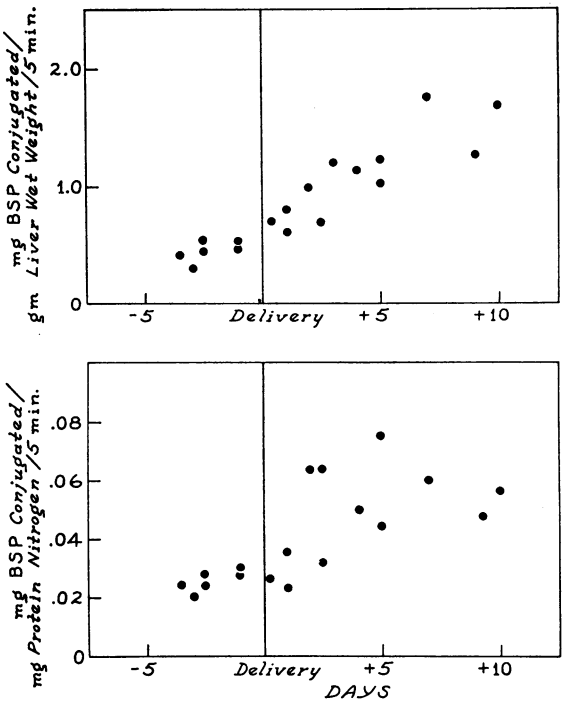

FIG. 2. BSP-GLUTATHIONE CONJUGATING ENZYME ACTIVITY IN RAT LIVER RELATED TO AGE. Enzyme activity is expressed per gram of liver wet weight (upper half of figure), and per milligram of protein nitrogen (lower half). Each point represents the value for a pool of livers obtained from litter mates. These values are not also included in Figure 1. They were obtained on additional animals to demonstrate that the increase in enzyme activity after birth occurs whether enzyme activity is expressed per gram wet weight of liver or per milligram of protein nitrogen.

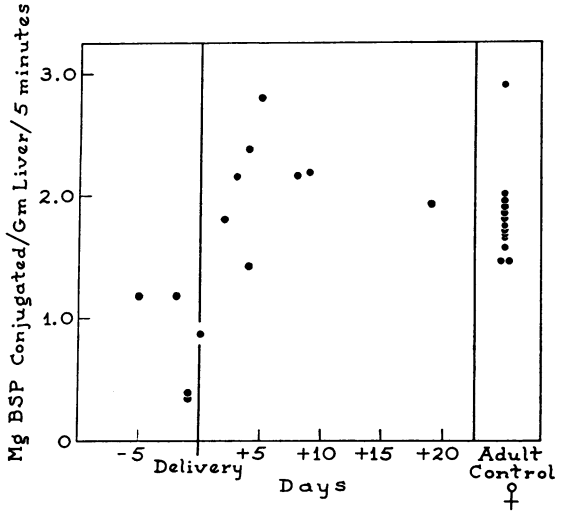

FIG. 3. CHANGES IN BSP-GLUTATHIONE CONJUGATING ENZYME ACTIVITY IN MATERNAL RAT LIVER BEFORE AND AFTER DELIVERY. The vertical line at the left of the figure represents the expected date of delivery for animals sacrificed before term, and the date of delivery for those sacrificed post partum. The adult control females had never been pregnant.

male rats 9 weeks of age and older, weighing 166 to 409 g, averaged 1.96 mg per g liver wet weight. Glutathione levels in fetal liver obtained on days 19 and 20 of gestation were approximately 50 per cent of the average adult value. There was little change in glutathione concentration during the first 3 weeks after delivery. Subsequently, however, it increased into the adult range. Glutathione levels measured in several maternal livers

TABLE II

BSP-glutathione conjugating enzyme activity in mixtures of fetal and adult liver homogenates

Source of liver	Liver in 1 ml homogenate	BSP conjugated in 5 minutes by 1 ml homogenate	BSP conjugated in 5 minutes by mixed homogenates† Expected
	mg	*mg*	*mg*
Fetus 1*	250	0.110	
Fetus 2*	250	0.118	
Adult 1	40	0.118	
Adult 2	40	0.102	
Fetus 1+Adult 1	†	0.110	0.114
Fetus 1+Adult 2	†	0.107	0.106
Fetus 2+Adult 1	†	0.118	0.118
Fetus 2+Adult 2	†	0.078	0.110

* Fetuses were obtained during day 21 of the gestation period. Livers from 4 litter mates in each group were pooled.

† Mixtures of homogenates consisted of 0.5 ml of fetal + 0.5 ml of adult.

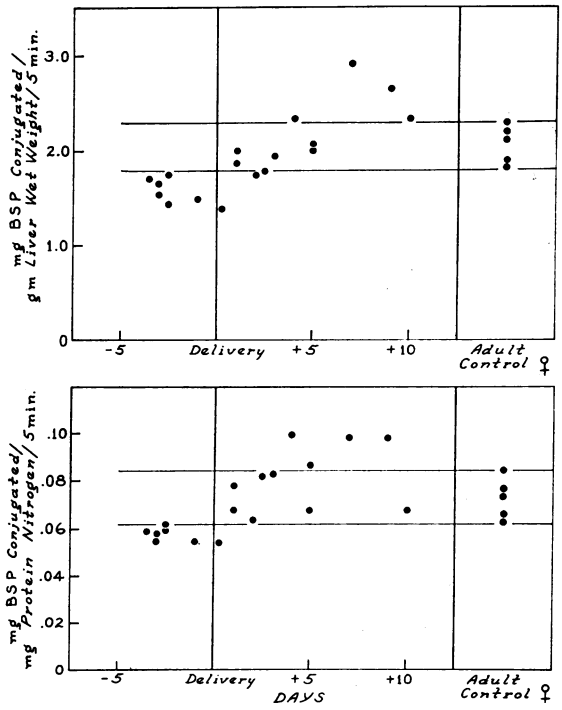

FIG. 4. BSP-GLUTATHIONE CONJUGATING ENZYME ACTIVITY IN MATERNAL RAT LIVER BEFORE AND AFTER DELIVERY. Enzyme activity is expressed per gram of liver wet weight (upper half of figure), and per milligram of protein nitrogen (lower half). The data in this figure were obtained from a different group of animals from those used for Figure 3.

obtained before and after delivery appeared to remain within the range found in adult rats.

DISCUSSION

The two components of the BSP-glutathione conjugating system normally found in liver—namely, conjugating enzyme and glutathione—were shown to be present in decreased amounts during the neonatal period. They increased soon after delivery, and within a relatively short period after birth approached adult levels. The changes in enzyme activity were much more striking than the alterations in glutathione concentration. Thus, hepatic enzyme activity, initially very low in the few days before birth, increased 2.5- to 10-fold by the third postpartum day, from values approximately 5 to 20 per cent to 50 per cent of adult activity. After this very rapid increase, conjugating enzyme activity continued to rise more gradually, reaching adult levels by about 5 to 7 weeks

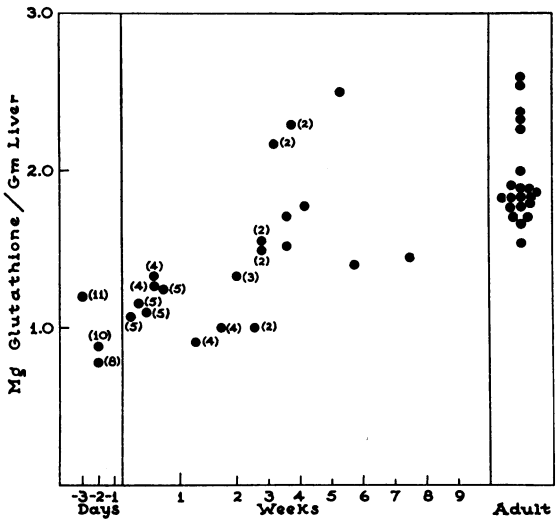

FIG. 5. RELATIONSHIP OF GLUTATHIONE CONCENTRATION IN RAT LIVERS TO AGE. Each point represents the value for either a pool of livers obtained from litter mates or for a single animal. The number of animals in each pool is listed in parentheses adjacent to the pertinent pool. Male and female rats are included in the pools up to 4 weeks of age. Single points from the third week on represent male rats.

post partum. By contrast, glutathione concentration in liver, although decreased in the neonatal period, was approximately 50 to 60 per cent of adult levels in the few days prior to delivery and changed only slightly during the first 3 weeks after birth. Thereafter, glutathione levels increased to adult values.

The present studies provide no direct explanation for the low conjugating enzyme activity found in fetal liver and the rapid increase in activity observed during the first few days after delivery. Mixing fetal and adult liver homogenates did not result in depression or enhancement of the expected average enzyme activity, tending to exclude the presence of an enzyme inhibitor in fetal liver or an enzyme activator in adult liver. The possibility of substrate induction of the enzyme cannot be resolved with certainty, since the identity of other physiological substrates in addition to glutathione is unknown. It is obvious that the increase in hepatic enzyme activity occurred in the absence of any exposure of the newborn animals to BSP. Although BSP was used to assay enzyme activity in these studies, we have demonstrated that the enzyme is not specific for BSP since it will catalyze the conjugation of glutathione with other compounds (18). In general, these are predominantly halogenated compounds that have been shown to be excreted as mercapturic acids (19). Although such compounds are exogenous, an as yet unknown endogenous compound that conjugates with the sulfur group of glutathione may be a physiological substrate for the enzyme. Further speculation about changes in hepatic content of such a hypothetical compound during the neonatal period, however, is unwarranted. It is possible that glutathione acted as a substrate inducer of increased enzyme activity. If so, it is striking that conjugating enzyme changed very little prior to delivery but changed markedly in the first few days post partum, despite a relatively constant concentration of glutathione in this period. Nevertheless, enzyme activity increased to approximately 50 per cent of adult levels, when glutathione concentration was about 50 to 60 per cent of adult values. Thereafter, liver glutathione concentration and BSP-conjugating enzyme activity appeared to increase proportionately until adult values were reached.

Of considerable interest is the observation that conjugating enzyme activity of maternal liver was decreased in the last trimester of pregnancy. Furthermore, after delivery, maternal enzyme activity rapidly increased and within a few days reached and even appeared to overshoot control values. These findings, in addition to the changes observed in fetal enzyme activity, suggest the presence of some inhibitory factor in pregnancy that decreases or disappears at the time of delivery, resulting in depression of conjugating enzyme activity of both the maternal and fetal liver during pregnancy and rapid increase in activity after birth. Recently, Lathe and Walker have presented evidence for the presence of a factor in human pregnancy sera that inhibits conjugation of bilirubin by liver slices (20). This factor increases during pregnancy, reaching a peak during the last trimester, then decreases after delivery. It is also found in fetal sera, but in lower concentration than in maternal sera, suggesting a maternal origin. After birth, the concentration of this factor in infant sera declines rather rapidly. Various steroids in concentrations approaching the physiological range were also found to inhibit

bilirubin conjugation by liver slices (20), suggesting that the inhibitory substances present in pregnancy sera might be steroid hormones produced in abundance during pregnancy. Although the nature of the inhibitor of bilirubin conjugation is still unknown, one can speculate that such an inhibitor may also be responsible for decreased BSP-conjugating activity of maternal and fetal liver during pregnancy.

Our current findings suggest that decreased BSP metabolism followed by a gradual increase may account, at least in part, for the delayed, then improved, BSP clearance from blood observed in the neonatal period by others (1, 3–7). Implicit in such a suggestion is that conjugation of BSP exerts a significant influence on the rate of BSP uptake by liver cells. It is currently believed that hepatic removal of BSP from plasma depends on the simultaneous operation of at least two separate processes: *1*) uptake of BSP in liver cells in an amount proportional to plasma concentration, and *2*) active secretion into bile (21–28). Conjugation of BSP with glutathione is not obligatory for hepatic BSP uptake, since appreciable quantities of free BSP may be found in the liver within minutes after intravenous administration (29). In addition, since free BSP has been regularly identified in bile (8–13), it is apparent that conjugation of BSP is not a prerequisite for biliary excretion. Nevertheless, once taken up in the liver, a major portion of the BSP is metabolized; over 50 per cent of the BSP recovered in liver 5 to 20 minutes after administration was shown to be conjugated (29). Furthermore, metabolized BSP accounts for most of the BSP excreted into bile in various species (8–10, 29). If conjugated BSP can be stored in liver or transported into bile to a greater extent than free BSP, metabolism of this compound may permit increased hepatic uptake. In preliminary studies we have been unable to demonstrate that conjugation influences the extent to which BSP is stored in the liver (30). However, observations of Philp, Grodsky and Carbone (29) and in our own laboratory (30) suggest that conjugated BSP can be excreted into bile at a faster rate than free BSP. Alterations in other factors that may affect hepatic uptake of BSP in the neonatal period, such as *1*) improved hepatic circulation, *2*) development of the transport mechanisms concerned with BSP movement across the hepatic cell membrane at both the sinusoidal and canalicular surfaces, and *3*) changes in the quality and quantity of protein in liver cells that may be involved in intrahepatic binding and storage of BSP, were not assessed and were by no means excluded by the present studies.

The present data are probably also relevant to mercapturic acid synthesis in the neonatal period. As mentioned previously, the liver enzyme assayed in the current studies is not specific for BSP because it will also catalyze the conjugation of glutathione with compounds such as benzyl chloride, bromobutane, *p*-fluoronitrobenzene, and others known to be excreted, at least in part, as mercapturic acids (19). Since it is likely that conjugation with glutathione is the first step in the formation of mercapturic acids (31, 32), the findings of low enzyme activity and low glutathione levels in liver suggest that mercapturic acid synthesis will be impaired in the newborn.

SUMMARY AND CONCLUSIONS

The BSP-glutathione conjugating system was examined in livers obtained from rat fetuses, *in utero,* and at various stages of development after delivery. BSP-glutathione conjugating enzyme activity of fetal liver was very low in the few days before birth, then rapidly increased 2.5- to 10-fold to approximately 50 per cent of adult activity by the third postpartum day. Thereafter it increased gradually, reaching adult levels by 5 to 7 weeks after delivery. Conjugating enzyme activity in maternal liver was also depressed prior to term, then rapidly increased soon after delivery. The concurrent depression of fetal and maternal enzyme activity during the latter part of gestation, followed by a rapid increase in activity in the early postpartum period, suggests the presence of an enzyme inhibitor in pregnancy that disappears after delivery. No evidence was found of an enzyme inhibitor in fetal liver or of enzyme activator in adult liver.

Hepatic glutathione concentration of young rats was reduced to approximately 50 to 60 per cent of adult levels in the few days before and for 3 weeks after delivery. Thereafter it reached adult levels. Glutathione levels of maternal liver obtained before and after term remained within the adult range.

The data suggest that delayed BSP clearance from blood observed in the human premature and full-term infant may be due, at least in part, to inadequate development of the BSP-glutathione conjugating system. Improved BSP clearance with increasing age could be explained by maturation of the conjugating mechanism.

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