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# Endogenous immunoreactive digitalis-like substance in neonatal serum and placental extracts

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## Summary

Therapeutic levels of digoxin in the serum of untreated neonates delivered to mothers who had not received the drug prenatally were detected by radio-immunoassay. Digoxin levels in neonates should be interpreted with care because of the unknown contribution by the endogenous digitalis-like substance (DLS) to the level of the drug.

Three commercially available radio-immunoassay kits were compared with regard to their sensitivity and reproducibility in detecting the endogenous DLS. The kit from Clinical Assays (Cambridge, Mass., USA) was selected for further investigations. In a series of 35 paired samples of maternal and cord blood the average DLS values in terms of digoxin were 0,52 ± 0,07 and 0,81 ± 0,27 ng/ml respectively. This difference is statistically highly significant. In the case of infants with DLS values of 1 - 1,5 ng/ml in terms of digoxin, approximately 1 week was required to reach non-therapeutic digoxin levels, i.e. below 0,5 ng/ml.

Gel chromatography showed that the DLS in neonatal serum was more closely associated with protein than is authentic digoxin. In placental extracts it followed the elution profile of the protein completely, but it shifted to fractions with a lower molecular weight than haemoglobin after trypsinization. The level of DLS in neonatal serum was also increased by more than half its original value by trypsinization. Proteolysis therefore seems to have a releasing effect on DLS. The molecular size of this substance is probably in the same range as that of polypeptides, since it was not dialysable from trypsinized and untreated samples through a membrane with a 22 000 dalton molecular weight cut-off point.

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The existence of an endogenous digitalis-like factor has received much attention in the recent literature.[1] The cardiac glycosides lend themselves to these investigations, since their concentrations can be determined with great specificity by radio-immunoassay and their high-affinity inhibitory action on $Na^+$-$K^+$-adenosine

triphosphatase ($Na^+$-$K^+$-ATPase) can be used as an indicator of biological activity. Kim and La Bella[2] have recently reviewed a number of substances which are possible candidates for the role of endogenous ligands for the digitalis receptor. In addition, a number of other laboratories have reported immunoreactive digitalis-like substances (DLSs) in the plasma and urine of volume-expanded dogs[3] and human volunteers[4] and in the serum of rats with experimental cardiac overload.[5] Digitalis immunoreactivity has also been found in extracts of mammalian brain[6] and rat adrenal glands.[7] It has also been shown that levels of a DLS are elevated in experimental hypertension and in hypertension in humans.[8,9] We have found therapeutic levels of digoxin-like immunoreactivity in neonates delivered to mothers who had not received the drug prenatally. This finding[10] has been confirmed by other independent studies[11,12] and has immediate clinical implications. In this report we wish to document the results of a comparison of three different commercially available radio-immunoassay kits for digoxin as well as some aspects of the molecular behaviour of the DLS.

## Material and methods

### Digoxin radio-immunoassays

Blood samples were collected in glass test tubes, and serum was obtained immediately after arrival of the blood sample at the laboratory. Three commercially available kits were used, Gammacoat (Clinical Assays, Cambridge, Mass., USA), Dac-Cel (Wellcome Diagnostics, Dartford, UK) and Amerlex (Amersham, Bucks, UK). The assays were performed in accordance with the manufacturers' instructions. A Packard Autogamma counter was used to determine the radioactivity of the $^{125}I$-labelled digoxin.

### Gel chromatography of serum and placental extracts

Gel chromatography on Sepharose 6B was performed according to standard procedures (further detail is provided in the legends to Figs 4 and 5).

Placental extracts were prepared by homogenizing the tissue at a ratio of 1 g per ml 0,02M tris-HCl, 0,1M NaCl (pH 8,0) in a Waring blender at full speed for 3 minutes. Tissue debris and microsomes were removed by successive centrifugations for 10 minutes at 30 000 *g* and for 1 hour at 100 000 *g*.

The elution buffer used was 0,1M ammonium acetate adjusted to a pH of 8 with dilute NaOH. The use of this buffer allowed direct lyophilization of the pooled fractions of which the digoxin or DLS levels were to be determined.

### Trypsinization of serum and placental extracts

Trypsin (Sigma, type 1) was added to serum and placental extracts to a final value of 1% of the mass of protein present in the sample. For placental extracts the latter was determined by the standard method of Lowry.[13] Proteolysis was allowed to take place at 37°C in a waterbath. The reaction was terminated by the addition of soybean trypsin inhibitor (Miles Seravac). A two-molar excess of inhibitor over enzyme was used. This procedure had no effect on the radio-immunoassay for authentic digoxin dissolved in buffer.

### Dialysis experiments

One-millilitre aliquots of neonatal serum (≈ 1 ng/ml DLS) and placental extracts (trypsinized and untreated) were dialysed against a 200-fold excess of 0,02M tris-HCl, 0,1M NaCl (pH 8,0) in a Schleicher and Schüll collodion thimble with a molecular weight cut-off point of 22 000 daltons. The protein concentration was determined by the Lowry method[13] in each sample assayed for DLS in order to correct for dilution which occurred because of volume expansion during dialysis.

## Results

### Commercially available radio-immunoassay kits for digoxin and DLS

In order to compare differences between DLS values in mothers and infants, 30 pairs of venous and cord blood samples were collected at random from mother-baby pairs who had not received digoxin at any stage. We used three different commercially available radio-immunoassay kits for the comparison. The results are summarized in Fig. 1. DLS levels are reported in ng/ml in terms of digoxin according to the standardization of the different kits with digoxin. We attach no other quantitative meaning to these levels except as an indication for immunoreactive interference. All the analyses with each kit reported in Fig. 1 were done in one batch. It is clear that all three kits showed the DLS values for the infants to be consistently higher than those for the mothers.

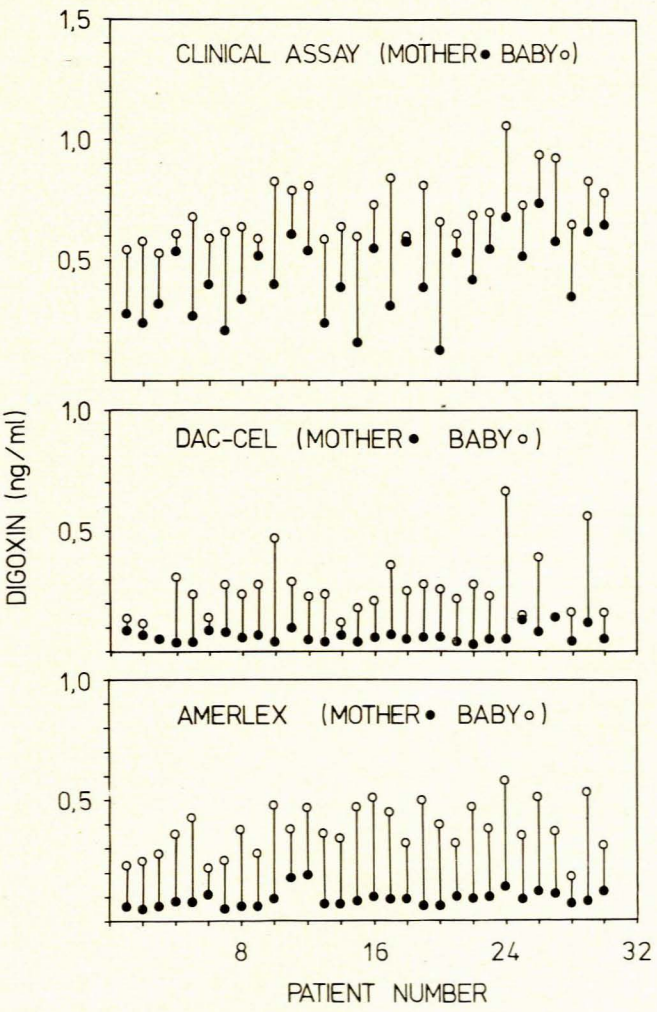


**Fig. 1. DLS in paired samples from mothers and their infants who had not received digoxin therapy, as determined with three different commercially available radio-immunoassay kits for digoxin. The levels relate to the standardization of the kits with authentic digoxin.**

The Dac-Cel and Amerlex kits consistently gave lower values for both mothers and infants than did the Clinical Assays kit. In Fig. 2 the relationship between the values obtained for neonates with the three respective kits is shown. The Clinical Assays kit gave the highest values, while the Amerlex kit gave intermediate values and the Dac-Cel kit the lowest values. A 1 ng/ml standard digoxin sample was interspersed after every fifth sample in the sequence, being analysed as a control. The Clinical Assays kit exhibited a coefficient of variation of only 2,75%, while those of the Amerlex and Dac-Cel kits were found to be 10,06% and 10,1% respectively. In addition to its greater accuracy, it is clear from Fig. 2 that the Clinical Assays kit has greater sensitivity to small differences in the lower ranges of detection of DLS (0,5 - 1 ng/ml).

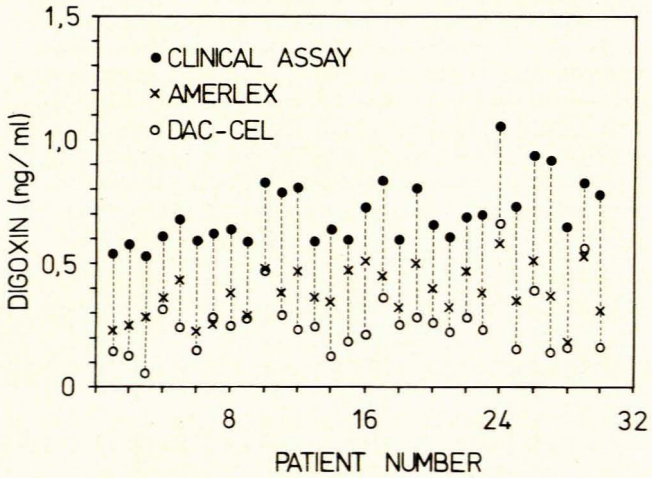


**Fig. 2. Serum DLS levels of neonates who had not received digoxin therapy determined with three different commercially available radio-immunoassay kits for digoxin (see Fig. 1).**

## DLS and clinical data

Levels of DLS in a further paired set of 35 randomly collected mother-baby samples were compared with clinical data such as age of mother, sex of baby, Apgar count, duration of pregnancy and method of delivery. Since a digoxin value of 0,5 ng/ml is considered clinically non-therapeutic and since the assay is not accurate for digoxin levels below 0,5 ng/ml, all the values below this were reported as 0,5 ng/ml. Using this value for the purposes of statistical calculations, the average level of DLS for the mothers was found to be $0,52 \pm 0,07$ ng/ml while that for the infants was $0,81 \pm 0,27$ ng/ml. This difference is statistically highly significant ($P < 0,001$; Student's paired $t$ test). No overall clear relationship was evident between DLS levels and the clinical features noted. However, the DLS level in 3 cases of pre-eclampsia was higher than average. The relationship between DLS and pre-eclampsia is described in the accompanying report (p. 883 of this issue of the *SAMJ*).

## DLS and age of the infants

Since all the samples analysed so far had been of cord blood, we investigated the kinetics of DLS in 2 infants who had been born with DLS levels higher than 1 ng/ml. Fig. 3 depicts the relationship between DLS levels and the age of the infants. Permission was granted by the Ethical Committee of the Faculty of Medicine to obtain heel-prick samples of capillary blood. It is clear that values approaching 0,5 ng/ml, i.e. clinically non-therapeutic digoxin values, were reached after about 1 week. We also measured the DLS levels of approximately one-third of the

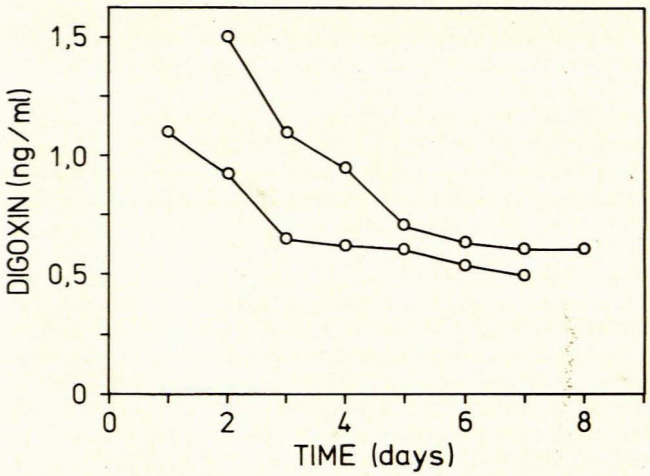


**Fig. 3. The relationship between serum DLS levels and age in 2 infants. The levels were derived by using a Clinical Assays kit standardized with authentic digoxin.**

35 infants described above after 6 weeks. All of them had levels below 0,5 ng/ml.

## Characteristics of the DLS in neonatal blood and placental extracts

In order to obtain information on the degree of protein association of the DLS compared with that of digoxin, sera from a neonate and from a patient receiving digoxin were chromatographed on Sepharose 6B. The elution profiles of the protein fractions in relation to the fractions which contained digoxin and DLS are shown in Fig. 4. Resolution of the plasma proteins according to size did not reveal separate distinct peaks. However, it is clear from Fig. 4 that the DLS eluted with the major protein peak and its descending limb. The largest part of this peak consists of serum albumin. Digoxin, on the other hand, was also associated with the descending limb of the serum albumin peak, but a large proportion eluted later, i.e. it represented unbound digoxin. The level of protein binding for digoxin is about 25%.[14]

In an effort to trace the origin of the DLS in neonatal serum, placental extracts from mothers whose infants had DLS levels of over 1 ng/ml in terms of digoxin at birth were made according to the procedure described under 'Methods'. The water-soluble extracts gave positive values for DLS and they were therefore chromatographed on Sepharose 6B in order to obtain information on the relative size of the molecule and its association with protein (Fig. 5).

From the upper half of Fig. 5 it is clear that the DLS in the placental extract is largely associated with the protein elution profile. We have subsequently treated the placental extract with trypsin and rechromatographed it on the same column (see the lower half of Fig. 5). Treatment with trypsin did not destroy the digitalis-like immunoreactivity, but it clearly shifted its elution pattern towards the lower molecular weight range (lower than that of haemoglobin, as indicated by its absorbance at 414 nm in Fig. 5).

The effect of trypsin on the level of DLS in neonatal serum was evaluated by analysing samples after increasing times of incubation with the enzyme. Control serum, buffer alone and serum from a patient on digoxin were also included in the experiment. The results are depicted in Fig. 6. The levels of both digoxin and DLS rose by 65-70% after 4 hours' incubation and thereafter remained constant. As in the case of placental extract the DLS in plasma appeared to be resistant to trypsinization. The substantial rise which occurred in the levels of both DLS

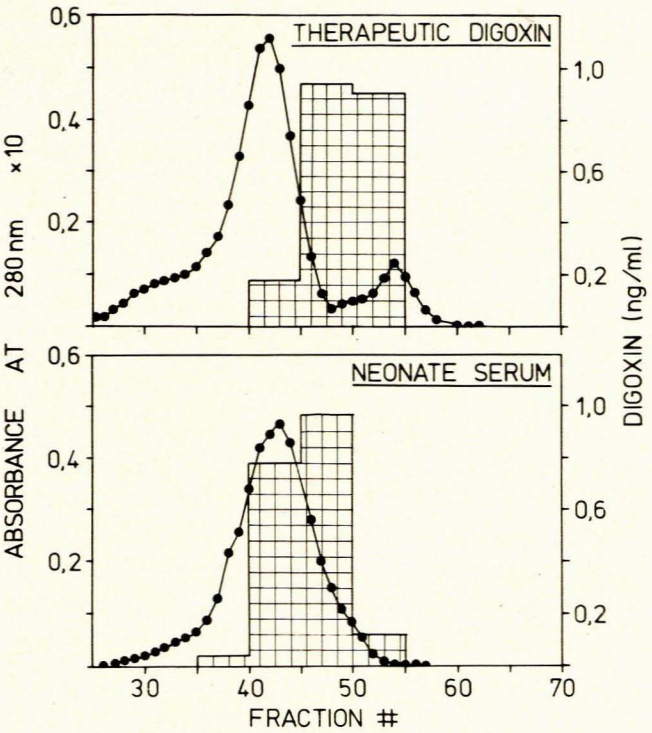


**Fig. 4. Comparison of the elution behaviour of authentic digoxin and DLS in neonatal serum by gel chromatography. A 1,5 x 90 cm column of Sepharose 6B was equilibrated with 0,1M ammonium acetate (pH 8,0) and 2 ml serum samples were loaded. Fractions of 2 ml each were collected from the moment of application. Pools of five fractions were lyophilized and reconstituted to 2 ml with 0,02M tris-HCl, 0,1M NaCl (pH 9,0), after which digoxin or DLS was determined with the Clinical Assays kit. Approximately 90% of the digoxin and DLS originally applied was recovered. The upper graph depicts the results obtained with a serum sample from a patient on digoxin therapy and the lower graph a serum sample from a neonate who had not received digoxin.**

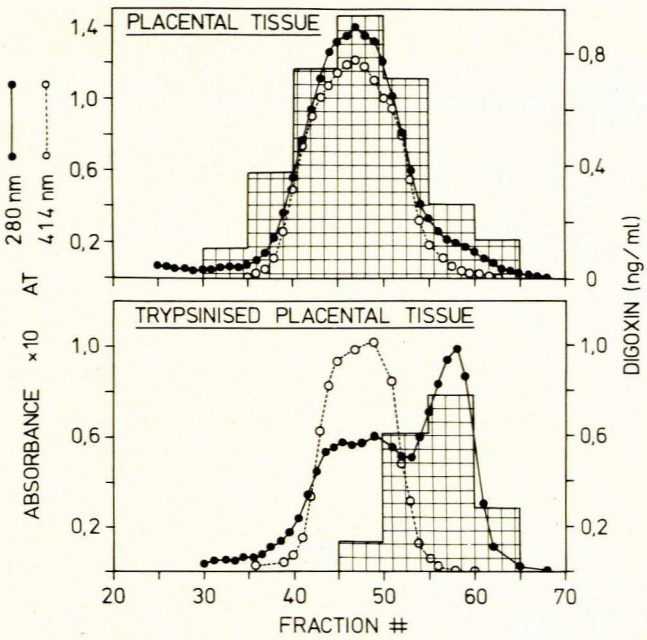


**Fig. 5. Gel chromatographic behaviour of DLS present in extracts from placental tissue, prepared as described under 'Methods'. Chromatographic procedures were similar to those described in Fig. 4. The upper graph depicts the profile obtained with a freshly made extract. The lower graph represents an extract incubated for 16 hours at 37°C with trypsin before chromatography. The trypsin concentration was 1% of the total protein. Samples of 2 ml containing 10 mg protein were loaded in each case. The absorbance at 414 nm indicates the elution profile of haemoglobin relative to the total protein which was measured at 280 nm.**

and digoxin may be due to decreased protein binding resulting from proteolysis. Although the levels in the control serum were very low in comparison with those in digoxin- and DLS-containing sera, a rise of about 50% was also noted after 4 hours of proteolysis. It therefore seems likely that normal serum contains a substance released by trypsinization which then interacts with the anti-digoxin antibodies. We do not know whether or not this substance is similar to the DLS found in neonatal serum and placental extracts.

In accordance with the high degree of protein association of DLS in neonatal serum (Fig. 4), it was found not to be dialysable through a collodion membrane with a 22 000 dalton molecular weight cut-off point. Appropriate corrections were made for the dilution of the serum caused by volume expansion during dialysis. No change in the level of DLS was found after 16 hours of dialysis at room temperature. The DLS level of trypsinized and untreated placental extracts was also not affected by dialysis. As a control we dialysed a serum sample from a patient on digoxin therapy who had a serum digoxin level of 3,6 ng/ml. After 16 hours of dialysis the level decreased to below 0,5 ng/ml.

## Discussion

The commercial availability of radio-immunoassay kits for digoxin makes therapeutic monitoring of this drug standard practice at most major centres. There is no doubt that this practice improves the quality of digoxin therapy to the benefit of the patient.[15]

The reliability of digoxin values reported by a laboratory depends upon the quality of the radio-immunoassay kit being used and the precision and care exercised by the operator. We have used three different kits to investigate the digitalis-like immunoreactive interfering substance. Fig. 2 shows clearly that each kit gave different values for the same sample. Specificity of the anti-digoxin antibodies may in part be responsible for the discrepancies observed between the kits. On a statistical basis, however, the Clinical Assays kit had the lowest coefficient of variation (2,75%) with an authentic 1 ng/ml sample of digoxin as standard. Despite the differences between the kits the levels of DLS in cord blood were always higher than the maternal levels with all three kits used (Fig. 1).

We were able to show that in neonates high levels of DLS in terms of digoxin returned to non-therapeutic levels approximately 7 days after birth (Fig. 3). This is in contrast to the finding of Valdes *et al.*,[12] who found that therapeutic levels of digoxin persisted for periods longer than 1 week.

The molecular nature of the endogenous digitalis-like factor observed by other workers has not been fully characterized. According to La Bella[1] and Schreiber *et al.*[16] the putative endogenous ligand for the digitalis receptor may include both steroids and peptides. On the basis of a positive fluorescamine reaction, Gruber *et al.*[3] concluded that it might be a small polypeptide. Although our study concentrates primarily on the interference of this substance with the radio-immunoassay for digoxin in serum, we have also employed gel chromatography and other techniques in an attempt to investigate some of the characteristics of the DLS. From Fig. 4 it is clear that this substance is more closely associated with the plasma proteins than is authentic digoxin. It was also not dialysable. The association of digitalis-like immunoreactivity with protein is even more striking when a soluble placental extract is chromatographed on Sepharose 6B. As indicated in Fig. 5 it follows the

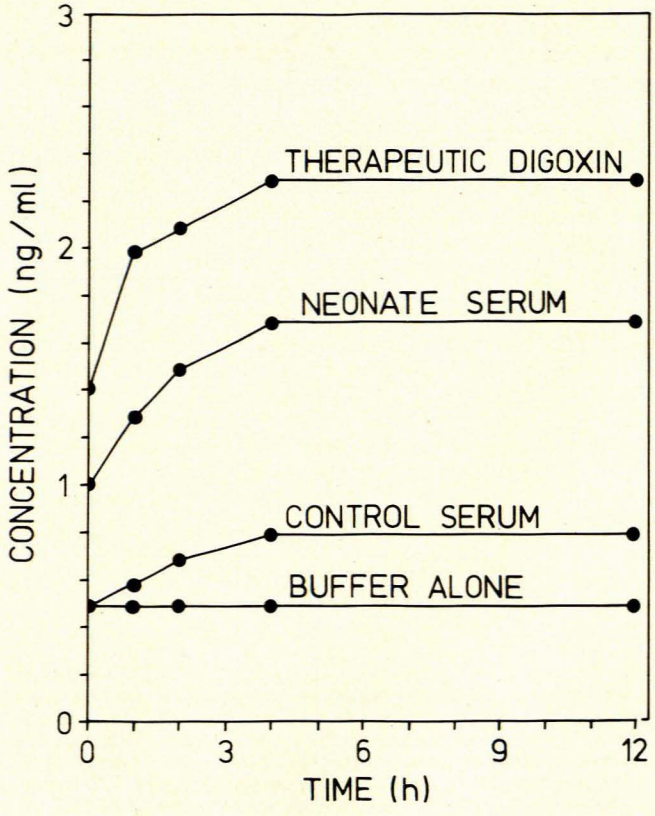


**Fig. 6. The effect of trypsinization on DLS and digoxin levels of serum. One millilitre samples were incubated with trypsin at 37°C. The enzyme concentration was 1% of the total protein. At each sampling time 200 μl aliquots were taken, soybean trypsin inhibitor was added (two-molar excess), and the DLS or digoxin level was determined with the Clinical Assays kit. The buffer control was 0,02M tris-HCl, 0,1M NaCl (pH 8,0), to which trypsin was added as in the serum samples.**

protein elution profile closely. The digitalis-like immunoreactivity can, however, be transformed to smaller molecular weight fractions by trypsinization. Like DLS in serum the immunoreactivity of the trypsinized placental material was also not dialysable.

Our experiments show that the immunoreactivity of this substance against anti-digoxin antibodies is not destroyed by trypsin. In fact it appears that the enzyme releases the DLS. Its concentration increased in neonatal blood incubated with trypsin (Fig. 6). We are utilizing this phenomenon as an aid in the isolation and purification of the DLS from placental tissue in an effort to correlate its immunological behaviour with some biological activity such as $Na^+$-$K^+$-ATPase inhibition. Trypsin also increased the level of digoxin in serum samples from patients on this drug (Fig. 6). This may partly be due to the destruction of protein by the enzyme, which means that less protein binding takes place. On the other hand it may also be partly due to increased digitalis-like immunoreactivity, since the level of the latter in serum from patients who were not on digoxin therapy also rose (Fig. 6). Therefore, apart from the DLS level in the therapeutic range for digoxin in neonatal blood the observation of increased immunoreactivity after proteolysis becomes important when digoxin therapy is monitored with a sensitive radio-immunoassay kit such as the one used by us.

In an independent study by Pudek *et al.*[11] on DLS in neonates, another commercial radio-immunoassay kit (NML Laboratories, Dallas, Texas, USA) was used which gave approximately double the values of the Clinical Assays kit. Their values for a series of 25 premature and full-term infants ranged from 0,3 to 4,1 ng/ml, with a mean of 1,4 ± 0,8 ng/ml. Our value of 0,81 ± 0,27 ng/ml roughly corresponds with this, if the sensitivity of the different kits for the interfering substance is taken into account. Pudek *et al.*[11] also attempted to correlate weight, medications and Apgar score with the DLS level of the infant. Like us, they did not find any significant correlation. We have, however, extended our series in order to investigate the possibility of a positive correlation between pre-eclampsia and DLS. These results are presented in the accompanying paper (p. 883).

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