Proc. Natl. Acad. Sci.USA
Vol. 75, No. 8, pp. 3909–3912, August 1978
Genetics

Developmental change in human intestinal alkaline phosphatase

(fetal/adult/electrophoresis/inhibition)

RICHARD A. MULIVOR, VICKIE L. HANNIG, AND HARRY HARRIS

Department of Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Contributed by Harry Harris, June 5, 1978

ABSTRACT **Starch gel electrophoresis and inhibition studies with L-phenylalanine, L-homoarginine, L-leucine, L-leucylglycylglycine, and L-phenylalanylglycylgylcine were carried out on a series of human alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1] derived from fetal and adult liver, kidney, bone, and intestine. No differences between adult and fetal liver, kidney, or bone alkaline phosphatases were observed by either electrophoretic or inhibition studies. However, the fetal intestinal enzyme could be clearly distinguished from the adult intestinal enzyme by its greater anodal electrophoretic mobility and its retardation after treatment with neuraminidase. Even after extensive neuraminidase treatment, its anodal mobility was still slightly greater than that of adult intestinal alkaline phosphatase. Fetal and adult intestinal enzymes showed the same inhibition profiles with the series of inhibitors both before and after treatment with neuraminidase. A survey of intestinal samples from fetuses and premature infants of various gestational ages indicated that the changeover from the synthesis of fetal to adult intestinal enzyme begins at about 28–32 weeks of gestation. The difference between the fetal and adult forms of intestinal alkaline phosphatase may represent the expression of different gene loci or a difference in post-translational modification.**

The human alkaline phosphatases are a group of similar enzymes with alkaline pH optima that hydrolyze a variety of monophosphate esters [orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1]. They are glycoproteins (1–3), and different forms occur in different tissues, the best characterized being those obtained from placenta, intestine, liver, bone, and kidney (4). Several lines of evidence suggest that at least three separate structural loci are concerned in determining the protein portion of the different alkaline phosphatase enzymes: one coding for the placental enzyme, a second for the intestinal enzyme, and at least one locus for the bone, liver, and kidney forms of the enzyme. (*a*) Several common electrophoretic variants of placental alkaline phosphatase have been identified and shown to be attributable to alleles at an autosomal locus (5). The intestinal, liver, bone, and kidney enzymes do not show such variants and are presumably determined by some other locus (or loci) (6). (*b*) The mutant gene that causes the rare autosomal recessive disease hypophosphatasia results in a gross deficiency of liver, bone, and kidney alkaline phosphatases, but does not effect the intestinal or the placental enzyme (7–9). (*c*) Purified placental and liver alkaline phosphatases differ in their peptide fingerprint patterns and in their amino-terminal amino acid sequences (10). (*d*) Quantitative inhibition studies with L-phenylalanine, L-homoarginine, L-leucine, L-leucylglycylglycine, and L-phenylalanylglycylglycine give identical inhibition profiles with the liver, bone, and kidney enzymes but sharply distinguish this group of enzymes from the placental and intestinal enzymes, which are also sharply distinguished from each other (11). These three classes of alkaline phosphatase may also be differentiated by electrophoretic studies both before and after treatment with neuraminidase, which removes sialic acid residues (6, 12–14), by thermostability studies (15, 16), and by immunological methods (3, 17–20).

The placental alkaline phosphatase phenotype is determined by the genotype of the fetus and not by that of the mother (5). Thus, the placental enzyme can be regarded as a "fetal" enzyme. In the work reported here we compare fetal samples of other tissues with corresponding adult samples in order to see whether further forms of alkaline phosphatase characteristic of fetal life and perhaps attributable to other loci could be identified.

MATERIALS AND METHODS

Samples. Adult liver, kidney, bone, and intestinal samples were obtained from autopsies generally conducted within 12 hr of death. Fetal tissue samples were obtained post mortem from induced elective abortions or at autopsy from babies delivered prematurely who expired shortly after delivery. Meconium samples (first stool of the newborn) came from an unselected series of births at the Hospital of the University of Pennsylvania. The tissue samples were stored at −20° if not used immediately.

Extraction was carried out by a modification of the butanol method of Morton (21). A small quantity of tissue was homogenized in the presence of an equal volume of water and half this volume of *n*-butanol with a Polytron homogenizer (Brinkman). The homogenate was incubated for 0.5 hr at 37° and then centrifuged (12,100 × *g*, 20 min, 5°). The intermediate aqueous layer was removed and used for the analyses.

Crude intestinal extracts usually contained, in addition to the characteristic intestinal alkaline phosphatase, a liver-like alkaline phosphatase. Prior to inhibition studies on these extracts, they were heated at 56° for about 90 min. This treatment results in the complete destruction of the liver-like enzyme while only 40–50% of the intestinal enzyme activity is lost. Electrophoresis after neuraminidase treatment established that the liver-like component was completely destroyed; in addition, further heating caused no additional change in the inhibition characteristics of the sample.

Electrophoresis. Starch gel electrophoresis of the tissue extracts was carried out at 5° using a Tris borate discontinuous buffer system, pH 8.0–8.6 (6). Neuraminidase treatment was performed by mixing a volume of sample (0.5 ml) with an equal volume of 10.0 mM Na_2HPO_4/citrate buffer, pH 5.0, containing one unit of neuraminidase (Sigma type VI), and incubating for 60 min at 37°.

Enzyme Assays and Inhibition Studies. Alkaline phosphatase activity was determined at 30° in a 1.0-ml reaction mixture containing 5.0 mM *p*-nitrophenyl phosphate (Sigma), 1.0 M diethanolamine (Fisher Scientific Company) (pH 9.8), 0.28 M NaCl, and 0.5 mM $MgCl_2$. The assay was stopped by addition of 1.0 ml of 1.0 M Na_2HPO_4 adjusted to pH 10.2 with 1.0 M NaOH.

Absorbance was determined at 405 nm in a Gilford model

2400S spectrophotometer equipped with a Haake circulating waterbath maintained at 30°. Each assay was performed in at least triplicate and was read against a blank containing the complete reaction mixture including enzyme which was stopped at zero time.

The same conditions were used for determining the relative activity in the presence of various inhibitors except for the inclusion of a particular inhibitor in the reaction mixture. The inhibitors were assayed at the following concentrations: L-phenylalanine, 2.5 mM, L-homoarginine, 10 mM; L-leucine, 5 mM; L-leucylglycylglycine, 5 mM; and L-phenylalanylglycylglycine, 1 mM. All amino acids and tripeptides with the exception of L-homoarginine (Calbiochem) were purchased from Sigma Chemical Company.

RESULTS

Intestinal alkaline phosphatase

Intestinal alkaline phosphatases from six fetuses (five of 20 weeks gestation and one of 24 weeks), obtained from induced elective abortions, were compared by electrophoresis with a series of samples of adult intestinal alkaline phosphatase. Two consistent differences between the fetal and adult intestinal enzymes were seen. In each case the native fetal intestinal enzyme had a faster anodal migration than the adult intestinal enzyme. Furthermore, after treatment with neuraminidase, the mobility of the fetal intestinal enzyme was reduced in contrast to that of the adult intestinal enzyme, which is not affected by neuraminidase (Fig. 1). Even after very extensive neuraminidase treatment (3 times the usual amount of neuraminidase and up to 19 hr of incubation), the fetal intestinal enzyme showed a slightly faster anodal mobility than the adult intestinal enzyme. These findings suggest that the fetal intestinal enzyme contains sialic acid residues not present in the adult intestinal enzyme, and that even after their removal some molecular difference between the two forms remains.

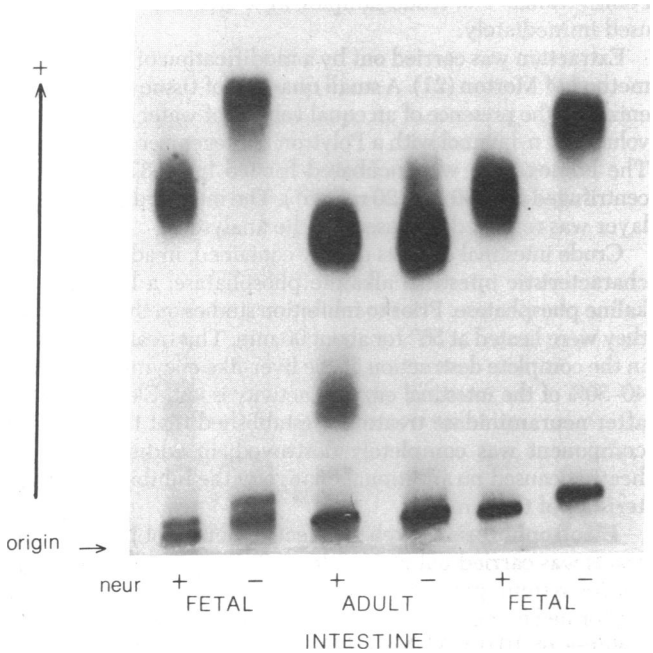

FIG. 1. Electrophoretic separations of adult and two different samples of fetal intestinal alkaline phosphatase before (−) and after (+) treatment with neuraminidase. Electrophoresis was carried out at pH 8.6. The gels were stained for enzymatic activity. Extracts of adult intestine show, in addition to the characteristic intestinal enzyme, an additional enzyme component resembling liver/bone alkaline phosphatase in its properties. Before neuraminidase treatment this is seen as a diffuse zone migrating more anodally than the characteristic adult intestinal enzyme and as a much less anodally migrating zone after neuraminidase treatment. The alkaline phosphatase bands near the origin are high molecular weight aggregates of the main enzyme bands that have reduced mobilities in starch gel.

In order to find out when the changeover from the fetal to the adult type of intestinal alkaline phosphatase occurs, we examined a further series of 21 intestinal samples obtained from autopsies on babies delivered prematurely and who died shortly after delivery and from full-term infants who died shortly after birth. The findings are summarized in Table 1. The alkaline phosphatases from the different samples were classified on the basis of the electrophoretic findings as fetal in type, adult in type, or mixed. The so-called mixed samples both before and after neuraminidase treatment showed a diffuse zone of activity overlapping the positions taken up by the control fetal and adult intestinal enzymes. The results suggest that the changeover from synthesis of fetal type to adult type intestinal alkaline phosphatase starts between about 28 and 32 weeks gestation.

Table 2 summarizes the inhibition results on ten different samples of adult intestinal alkaline phosphatase and eight samples of the fetal intestinal enzyme. The inhibition profiles for this battery of inhibitors are essentially the same for both the fetal and adult forms of the enzyme. There is marked inhibition with L-phenylalanine, modest inhibition with L-leucine, and only slight inhibition with L-homoarginine, L-leucylglycylglycine, and L-phenylalanylglycylglycine at the concentrations of inhibitors used. The inhibition profile is clearly distinct from that obtained from bone, liver, kidney, or placental alkaline phosphatases (11).

Inhibition results on 20 samples of meconium alkaline phosphatase are also given in Table 2. Other studies have shown that meconium, the first stool of the newborn, is rich in the enzyme, and we have found that this is very similar to the fetal intestinal enzyme in its electrophoretic properties both before and after neuraminidase treatment. As shown in Table 2, the meconium enzyme has essentially the same inhibition profile as the fetal and adult intestinal enzymes.

In order to learn whether removal of the sialic acid residues affected the inhibition characteristics of the fetal intestinal or meconium alkaline phosphatase, we examined a sample of each before and after neuraminidase treatment and compared them to a sample of the adult intestinal enzyme treated in a similar manner. Each sample (0.1 ml) was mixed with 0.5 ml of 5.0 mM Na_2HPO_4/citrate buffer, pH 5.0 containing one unit of neuraminidase and incubated for 1 hr at 37°. At the same time, a control sample (0.1 ml) was mixed with 0.5 ml of the same buffer as the treated sample, only lacking the neuraminidase, and was also incubated for 1 hr at 37°. After incubation, the samples were dialyzed against 10 mM Tris·HCl, pH 7.5/1.0 mM $MgCl_2$. The results of the inhibition studies both with and without neuraminidase treatment are shown in Table 3. There

Table 1. Changeover from fetal to adult alkaline phosphatase

Wk after conception	No. of individuals		
	Fetal	Mixed	Adult
20–25	6*	0	0
26–30	4	1†	0
31–35	1‡	1§	1§
36–40	0	3	4
>40	0	0	6

* Elective abortion.
† 28 weeks.
‡ 32 weeks.
§ 35 weeks.

Table 2. Inhibition of intestinal alkaline phosphatase

Intestinal enzyme	L-Phe (2.5 mM)	L-Har (10.0 mM)	L-Leu (5.0 mM)	L-LeuGlyGly (5.0 mM)	L-PheGlyGly (1.0 mM)
Adult (10)	23.0 ± 1.2	80.0 ± 2.2	37.8 ± 0.8	79.8 ± 2.3	76.4 ± 2.6
Fetal (8)	22.1 ± 0.7	79.3 ± 1.6	36.5 ± 0.8	78.7 ± 1.8	75.5 ± 1.1
Meconium (20)	23.7 ± 1.0	81.1 ± 1.5	38.3 ± 1.4	80.8 ± 1.9	77.6 ± 1.6
Combined (38)	23.2 ± 1.4	80.4 ± 1.9	37.8 ± 1.4	80.1 ± 2.1	76.9 ± 1.9

Inhibition is expressed as the mean percentage of original activity remaining (±SD) in the presence of the inhibitor. Number of samples is given in parentheses.

are no significant differences in the percentages of activity remaining for any of the inhibitors between the samples treated with neuraminidase and the corresponding untreated samples. Total enzyme activity for each sample both before and after neuraminidase treatments was also determined, and no differences attributable to neuraminidase treatment were apparent. In order to check that the treatment with neuraminidase had been complete, we examined the paired samples by starch gel electrophoresis. The electrophoretic mobilities observed were precisely those expected and indicated that the removal of the sialic acid residues had gone to completion.

Liver, bone, and kidney alkaline phosphatases

Liver, bone, and kidney samples obtained from three fetuses of 20 weeks gestation (induced elective abortions) were compared by electrophoresis with a series of corresponding adult samples. No differences were noted between the electrophoretic patterns of the fetal and adult samples of the same tissue either before or after treatment with neuraminidase.

The electrophoretic patterns of liver, bone, and kidney alkaline phosphatases in the native state differ one from another; the bone and kidney enzymes show more diffuse zones of activity that migrate less anodally than the liver enzyme. However, after neuraminidase treatment there is marked retardation and the liver, bone, and kidney enzymes now show essentially the same mobilities. This suggests that the differences in electrophoretic mobility between the liver, bone, and kidney enzymes observed in the native state are largely, if not entirely, due to differences in their sialic acid contents.

Table 4 summarizes the inhibition results with the liver, bone, and kidney enzymes from the three fetuses and with a number of adult samples of alkaline phosphatase from these organs. The inhibition profiles were essentially the same for both the fetal and adult samples and for each of the tissues. Marked inhibition was obtained with L-homoarginine, modest inhibition with L-leucine and L-leucylglycylglycine, and only slight inhibition with L-phenylalanine and L-phenylalanylglycylglycine at the concentrations of the inhibitor used. It has been shown previously that treatment with neuraminidase does not affect the inhibition profile or activity of liver, bone, and kidney alkaline phosphatases (11).

DISCUSSION

Alkaline phosphatases from different fetal tissues were compared with those from corresponding adult tissues to see whether significant molecular differences occur. The methods used would have allowed us to distinguish enzyme molecules that differed in their total net charge before and after removal of sialic acid residues or that differed in their binding sites for a series of uncompetitive small molecular weight inhibitors.

No electrophoretic differences between fetal and adult alkaline phosphatases from liver, bone, and kidney were observed. All three enzymes from both fetal and adult tissues also gave essentially the same inhibition profiles with the series of inhibitors used. Thus, there is no suggestion of a further locus coding for any of these enzymes being expressed in fetal life, at least after about 20 weeks gestation.

In contrast, six samples of intestinal alkaline phosphatase obtained before 25 weeks gestation could be clearly distinguished from the adult intestinal enzyme by electrophoresis. They had a greater anodal mobility than the adult enzyme and also, unlike the adult enzyme, their electrophoretic mobility was retarded after treatment with neuraminidase. Further examples of this fetal intestinal enzyme were found in fetuses of greater gestational age, and the overall results suggest that the changeover from synthesis of the fetal form to the adult form starts to occur between 28 and 32 weeks of gestation.

The fetal and adult forms of intestinal alkaline phosphatase clearly differ in their content of sialic acid residues. However, even after these have been removed, some molecular difference appears to remain. Whether this is due to a further difference in the carbohydrate moiety or to a difference in the protein, which might imply coding by a separate gene locus, cannot be answered by simple electrophoretic analysis. The finding that the fetal and adult intestinal enzymes showed the same inhibition profiles indicates that the binding sites for the series of different inhibitors used are likely to be the same in the two classes of molecules.

It has been reported that the alkaline phosphatase in meconium has a different electrophoretic mobility from the adult intestinal enzyme (22), and Miki *et al.* (23) showed that it had a mobility similar to that of the intestinal enzyme from a stillborn fetus (6 months gestation). Furthermore, the electropho-

Table 3. Effect of neuraminidase on intestinal alkaline phosphatase

Enzyme	Neuraminidase	L-Phe (2.5 mM)	L-Har (10.0 mM)	L-Leu (5.0 mM)	L-LeuGlyGly (5.0 mM)	L-PheGlyGly (1.0 mM)
Adult	−	24.4	83.2	41.9	81.9	76.3
	+	24.8	81.6	42.5	81.3	75.7
Fetal	−	24.6	85.3	41.3	82.2	78.9
	+	24.0	82.2	40.8	81.2	76.2
Meconium	−	22.5	84.7	39.3	84.0	77.4
	+	25.3	83.5	42.1	82.1	80.2

Inhibition is expressed as the percentage of original activity remaining in the presence of the inhibitor.

Table 4. Inhibition of liver, bone, and kidney alkaline phosphatases

Enzyme	L-Phe (2.5 mM)	L-Har (10.0 mM)	L-Leu (5.0 mM)	L-LeuGlyGly (5.0 mM)	L-PheGlyGly (1.0 mM)
Liver					
Adult (9)	91.4 ± 2.1	20.4 ± 1.4	71.9 ± 1.2	60.8 ± 1.3	94.9 ± 0.9
Fetal (3)	90.9 ± 1.7	20.5 ± 0.6	72.8 ± 2.0	62.1 ± 0.4	94.9 ± 0.6
Combined (12)	91.3 ± 2.0	20.4 ± 1.2	72.2 ± 1.4	61.1 ± 1.3	94.9 ± 0.8
Bone					
Adult (12)	90.6 ± 2.2	21.8 ± 1.0	72.1 ± 1.7	61.0 ± 2.0	94.5 ± 2.2
Fetal (3)	92.4 ± 1.5	22.0 ± 0.7	72.7 ± 2.7	61.6 ± 2.5	95.0 ± 1.0
Combined (15)	91.0 ± 2.1	21.8 ± 0.9	72.2 ± 1.9	61.1 ± 2.0	94.6 ± 2.0
Kidney					
Adult (9)	88.8 ± 2.2	22.0 ± 2.1	70.6 ± 1.3	60.7 ± 1.5	93.8 ± 2.2
Fetal (3)	91.9 ± 1.5	20.1 ± 1.3	71.2 ± 1.0	60.1 ± 0.8	95.0 ± 1.1
Combined (12)	89.5 ± 2.4	21.5 ± 2.1	70.7 ± 1.2	60.6 ± 1.3	94.1 ± 1.3
Combined (39)	90.6 ± 2.3	21.3 ± 1.5	71.7 ± 1.7	60.9 ± 1.6	94.6 ± 1.5

Inhibition is expressed as the mean percentage of original activity remaining (±SD) in the presence of the inhibitor. Number of samples is given in parentheses.

retic mobility of meconium alkaline phosphatase was retarded by neuraminidase, though in a number of other properties it resembled the adult intestinal enzyme. We have confirmed this result in a series of 27 meconium samples and compared them with a series of fetal intestinal samples. It appears that the meconium enzyme is mostly fetal intestinal in type. It probably has its origin in desquamated fetal intestinal mucosa and progressively accumulates during the course of fetal life.

Thus, as late as 28–32 weeks of gestation an intestinal alkaline phosphatase different from that seen in the adult can be observed. The molecular basis of this difference is still uncertain, and it is not clear whether it represents the expression of different gene loci or whether it is due to some difference in post-translational modification. However, it is of interest that alkaline phosphatase resembling the fetal intestinal enzyme has been demonstrated in at least two reports of hepatocellular carcinoma (24, 25). Since a placental-like alkaline phosphatase has also been observed in various other malignant tumors (4, 26), both fetal forms of the enzyme may be aberrantly expressed in malignancy.

We thank Mr. L. Plotkin for his technical assistance and Drs. M. Mennuti (Department of Obstetrics and Gynecology), J. Wheeler (Department of Pathology, Hospital of the University of Pennsylvania), E. Zackai (Department of Human Genetics), C. Witzleben (Department of Pathology, Children's Hospital of Philadelphia), and Y. Edwards (Galton Laboratory, University College, London) for providing tissue samples essential to these studies. This work was supported by Grants VC-231 from the American Cancer Society, CA-20296, from the National Cancer Institute, Department of Health, Education and Welfare, PCM76-82997 from the National Science Foundation, and GM 00053 from the National Institutes of Health.

1. Ghosh, N. K., Goldman, S. S. & Fishman, W. H. (1967) *Enzymologia* **33**, 113–124.
2. Komoda, T. & Sakagishi, Y. (1976) *Biochim. Biophys. Acta* **438**, 138–152.
3. Komoda, T. & Sakagishi, Y. (1976) *Biochim. Biophys. Acta* **445**, 645–660.
4. Fishman, W. K. (1974) *Am. J. Med.* **56**, 617–650.
5. Robson, E. B. & Harris, H. (1967) *Ann. Hum. Genet. (London)* **30**, 219–232.
6. Harris, H. & Hopkinson, D. A., eds. (1976) *Handbook of Enzyme Electrophoresis in Human Genetics* (North Holland, Amsterdam).
7. Fraser, D. (1957) *Am. J. Med* **22**, 730–746.
8. Danovitch, S. H., Baer, P. N. & Laster, L. (1968) *New Engl. J. Med.* **278**, 1253–1260.
9. Mulivor, R. A., Mennuti, M., Zackai, E. & Harris, H. (1978) *Am. J. Hum. Genet.* **30**, 271–282.
10. Badger, K. S. & Sussman, H. K. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2201–2205.
11. Mulivor, R. A., Plotkin, L. I. & Harris, H. (1978) *Ann. Hum. Genet.* **42**, 1–13.
12. Robinson, J. C. & Pierce, J. E. (1964) *Nature (London)* **204**, 472–473.
13. Butterworth, P. J. (1966) *Nature (London)* **209**, 805–806.
14. Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1966) *Biochem. J.* **98**, 32c–33c.
15. Neale, F. E., Clubb, J. S., Hotchkis, D. & Posen, S. (1965) *J. Clin. Path.* **18**, 359–363.
16. Moss, D. W., Shakespeare, M. J. & Thomas, D. M. (1972) *Clin. Chim. Acta.* **40**, 35–41.
17. Boyer, S. H. (1963) *Ann. N. Y. Acad. Sci.* **103**, 938–951.
18. Sussman, H. K., Small, P. A. & Cotlove, E. (1968) *J. Biol. Chem.* **243**, 160–166.
19. Lehmann, F-G. (1975) *Clin. Chim. Acta* **65**, 257–269.
20. Khattab, M. & Pfleiderer, G. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 377–391.
21. Morton, R. K. (1954) *Biochim. J.* **57**, 595–603.
22. Hernández-Jodra, M. & Gancedo, C. (1971) *Enzyme* **12**, 682–687.
23. Miki, K., Suzuki, H., Iino, S., Oda, T., Hirano, K. & Sugiura, M. (1977) *Clin. Chim. Acta* **79**, 21–30.
24. Suzuki, H., Iino, S., Endo, Y., Torij, M., Miki, K. & Oda, T. (1975) *Ann. N. Y. Acad. Sci.* **259**, 307–320.
25. Higashino, K., Otani, R., Kudo, S. & Yamamura, Y. (1977) *Clin. Chem.* **23**, 1615–1623.
26. Fishman, W. H., Inglis, N. R., Stolbach, L. L. & Krant, M. J. (1968) *Cancer Res.* **28**, 150–154.