Proc. Natl. Acad. Sci. USA
Vol. 76, No. 8, pp. 4016–4019, August 1979
Genetics

Human cell lines expressing intestinal alkaline phosphatase

(inhibitors/thermostability/electrophoresis/HeLa cell/placental)

FRANCES J. BENHAM AND HARRY HARRIS

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

Contributed by Harry Harris, May 21, 1979

ABSTRACT **At least three loci determine human alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]: one coding for the placental form of the enzyme, at least one coding for the intestinal forms, and at least one for the liver, bone, and kidney forms. The alkaline phosphatase in cell line D98/AH-2 has been characterized by inhibition, thermostability, and electrophoretic studies. It is intestinal in type and resembles the fetal intestinal form somewhat more closely than the adult intestinal form. Intestinal alkaline phosphatase was found in the related cell lines Detroit 98, D98/S, and D98/AH-R. No placental alkaline phosphatase could be detected in any of these cell lines. This series of cell lines are believed, on the basis of earlier investigations, to be HeLa in origin but other HeLa cell lines show placental alkaline phosphatase. Loss of expression of the placental alkaline phosphatase locus probably occurred prior to the separation of Detroit-98 from the lineage leading to other HeLa cell lines and this has persisted in the Detroit-98 derivatives D98/AH-2, D98/S, and D98/AH-R. Another possibility is that placental alkaline phosphatase expression only appeared in the HeLa lineage subsequent to the separation of Detroit-98.**

Present evidence (summarized in refs. 1 and 2) indicates that in humans there are at least three gene loci that determine the different forms of alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1]: one coding for the placental form of the enzyme, at least one coding for the intestinal forms, and at least one coding for the liver, bone, and kidney forms. These three main classes of human alkaline phosphatase can be sharply discriminated one from another by inhibition (1, 3), thermostability (4–7), electrophoretic (8), and immunologic studies (9–12). In many cultured human cell lines the alkaline phosphatase has a low, though rather variable, activity and generally appears to be of the liver/bone/kidney type (13–15). Certain human cell lines, however, show relatively high alkaline phosphatase activity. In most cases these lines were originally derived from malignant tumors and are generally aneuploid. Alkaline phosphatase has been extensively studied in various sublines of HeLa, and a placental-like alkaline phosphatase has been shown to occur in a number of them (16–20). This has generally been interpreted as due to derepression or activation of the placental alkaline phosphatase locus.

HeLa was originally derived from a cervical carcinoma in the early 1950s. It has since been propagated in many different laboratories. Consequently, the various sublines have different cultural histories. In addition, studies of isozyme (21–23) and chromosomal markers (24–25) and also, most recently, endonuclease restriction enzyme analysis of ribosomal genes* have led to the conclusion that a number of long-term human cell lines, which were originally thought to have different origins, are in all probability HeLa lines that arose by contamination of the original line at some point in its history.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

In a recent study of the alkaline phosphatase in a number of different HeLa and presumptive HeLa sublines (20) we demonstrated, as expected, the presence of a placental-like alkaline phosphatase in most of the lines. However, in one cell line, D98/AH-2, although there was considerable alkaline phosphatase activity, its properties were quite different from placental alkaline phosphatase and no evidence for even small amounts of placental alkaline phosphatase was obtained. We have now investigated the characteristics of this alkaline phosphatase in some detail and conclude that it corresponds to intestinal alkaline phosphatase. Furthermore, it appears to resemble the fetal form of intestinal alkaline phosphatase more closely than the adult form.

MATERIAL AND METHOD

Cell lines D98/AH-2, D98/S, D98/AH-R, and Detroit-98 were obtained from the American Type Culture Collection. On arrival and thereafter at routine intervals, the cells were tested and found free of mycoplasmas. The cells were cultured as monolayers in plastic flasks with RPMI 1640 medium supplemented with 10% fetal calf serum. Cells at harvest were washed with EDTA and detached by a brief exposure to trypsin. The cell pellets were then thoroughly washed and stored at −20°C.

Alkaline phosphatase activity was assayed in sonicated cell homogenates as described (1), with 5.0 mM *p*-nitrophenylphosphate (pH 9.8). Total protein was measured by the method of Lowry *et al.* (26). Inhibition, thermostability, and electrophoretic studies were carried out on extracts prepared from cell homogenates by butanol extraction as described (20). Control tissue samples (liver, adult intestine, fetal intestine, and placenta) were obtained as reported (1, 2), and alkaline phosphatase solutions for analysis were prepared by butanol extraction. The intestinal samples were heated at 56°C for 90 min as described (1, 2), to destroy any liver-type alkaline phosphatase in the crude tissue extract. Inhibition studies were carried out as described (1), with *p*-nitrophenylphosphate (5.0 mM) as substrate and L-phenylalanine (2.5 mM), L-homoarginine (10 mM), L-leucine (5 mM), L-leucylglycylglycine (5 mM), and L-phenylalanylglycylglycine (1.0 mM) as inhibitors.

Thermostability studies were carried out on samples that had been dialyzed against 10 mM Tris·HCl, pH 7.4/5.0 mM $MgCl_2$. D98/AH-2 extracts were heated at 56°C for 90 min in the same way as the intestinal extracts. Aliquots of the enzyme solutions (0.1 ml) were heated in a waterbath at 60°C for 10 and 25 min and were then immediately transferred to an ice bath, in which unheated samples were maintained for the same times. Starch gel electrophoresis was carried out with a Tris/borate discontinuous buffer system (pH 8.0–8.6) as described (20), except that 1% Triton X-100 was added to the buffers. Neuraminidase

* Schmickel, R., Knoller, M., Szwia, L. & Wilson, G. (1978) American Society of Human Genetics 29th Annual Meeting, p. 121A (abstr.).

Table 1. Percent alkaline phosphatase activity remaining in the presence of various inhibitors

Inhibitor	Alkaline phosphatase controls, %			D98/AH-2, %
	Placental	Liver	Intestinal	
L-Phenylalanine (2.5 mM)	29.37 ± 1.15	92.30 ± 1.50	25.97 ± 0.75	24.71 ± 1.10
L-Homoarginine (10.0 mM)	84.56 ± 1.01	20.53 ± 0.73	80.47 ± 1.08	82.12 ± 1.36
L-Leucine (5.0 mM)	50.53 ± 1.49	72.91 ± 1.36	38.58 ± 0.94	38.47 ± 1.15
L-Leucylglycylglycine (5.0 mM)	14.14 ± 0.67	61.66 ± 1.21	80.41 ± 1.09	81.77 ± 1.24
L-Phenylalanylglycylglycine (1.0 mM)	8.93 ± 0.58	96.21 ± 1.00	80.11 ± 1.18	81.12 ± 1.56

D98/AH-2 alkaline phosphatase was compared with control placental, liver, and intestinal alkaline phosphatases. Means ± SEM are given.

treatment (*Clostridium perfringens*, Sigma, Type VI) of cell and tissue extracts was as described (20).

RESULTS

Activity Studies. The mean alkaline phosphatase activity in several preparations of the D98/AH-2 cell line was 676 nmol of *p*-nitrophenylphosphate hydrolyzed per min per mg of protein with a standard deviation of 66. This is about 50 times greater than the average activity in a series of cultured diploid human fibroblast lines.

Inhibition Studies. It was previously shown that there are no significant differences between liver, bone, and kidney alkaline phosphatases in the degree of inhibition obtained with the five inhibitors used here (1), nor are there significant differences in inhibition between adult intestinal and fetal intestinal alkaline phosphatases (2). However, the inhibitors do discriminate sharply between placental, intestinal, and liver/bone/kidney alkaline phosphatases (1).

Table 1 shows inhibitions obtained with D98/AH-2 alkaline phosphatase and with control placental, intestinal, and liver alkaline phosphatases. The percentage activities remaining in the presence of each of the inhibitors were essentially the same for D98/AH-2 and for intestinal alkaline phosphatase. However, they were clearly different from those of placental and liver alkaline phosphatases. Placental alkaline phosphatase was most marked differentiated from D98/AH-2 alkaline phosphatase by inhibition with L-phenylalanylglycylglycine and L-leucylglycylglycine, whereas liver alkaline phosphatase was most markedly differentiated by inhibition with L-homoarginine and L-phenylalanine.

Thermostability Studies. Placental alkaline phosphatase may be heated at 65°C for up to 60 min with no significant loss of activity (4). Under these conditions, liver, bone, kidney, and intestinal alkaline phosphatase are rapidly destroyed. At lower temperatures it can be shown that intestinal alkaline phosphatase is less thermolabile than liver, bone, and kidney alkaline phosphatases (7). D98/AH-2 activity was rapidly destroyed at 65°C, and at 60°C resembled intestinal alkaline phosphatase in thermolability (Table 2).

Table 2. Percent activity remaining after various alkaline phosphatases were heated at 60°C for 10 and 25 min

Alkaline phosphatase	% activity after 10 min		% activity after 25 min	
	n	Mean ± SD	*n*	Mean ± SD
Liver	10	6.5 ± 0.8	10	0.82 ± 0.03
Intestine				
Adult	9	73.2 ± 1.6	9	47.4 ± 4.0
Fetal	12	83.5 ± 3.5	11	67.1 ± 4.9
D98/AH-2	10	76.4 ± 5.1	8	58.2 ± 2.7

D98/AH-2 alkaline phosphatase was compared with liver, adult intestinal, and fetal intestinal alkaline phosphatases. *n*, Number of determinations.

Electrophoresis. In studies of alkaline phosphatase isozymes by starch gel electrophoresis at least two classes of components are usually seen in any tissue extract, one migrating relatively rapidly toward the anode and the other relatively slowly (8). The slowly migrating components are thought to represent high molecular weight aggregates of the faster moving components. Adult and fetal intestinal alkaline phosphatases can be differentiated from each other only by electrophoresis. The fetal intestinal components have faster anodal mobilities than the adult intestinal components. After treatment with neuraminidase, migration of the fetal components is retarded whereas that of the adult components is unaltered. However, even after desialation with neuraminidase, the fetal intestinal forms have slightly, though consistently, faster mobilities than the adult forms.

In our earlier studies of the alkaline phosphatases in HeLa cell lines, the multiple components present were difficult to resolve and were often very smeary. We have now found that considerably improved resolution is obtained if the electrophoresis is carried out in the presence of Triton X-100. Fig. 1 shows the patterns obtained after electrophoresis in the presence of Triton X-100 of placental, liver, adult intestinal, fetal intestinal, and D98/AH-2 alkaline phosphatases. The D98/AH-2 patterns both before and after treatment with neuraminidase closely resembled those of fetal intestinal alkaline phosphatase. They could be clearly distinguished from the patterns for adult intestinal, liver, and placental alkaline phosphatases and also, though not shown in Fig. 1, from bone and kidney alkaline phosphatases. Repeated comparisons of D98/AH-2 and various fetal intestinal alkaline phosphatase preparations have shown that the mobilities of the alkaline phosphatase components in D98/AH-2, though very similar to those of fetal intestinal alkaline phosphatase, do not match them exactly and generally appear to have slightly greater mobilities.

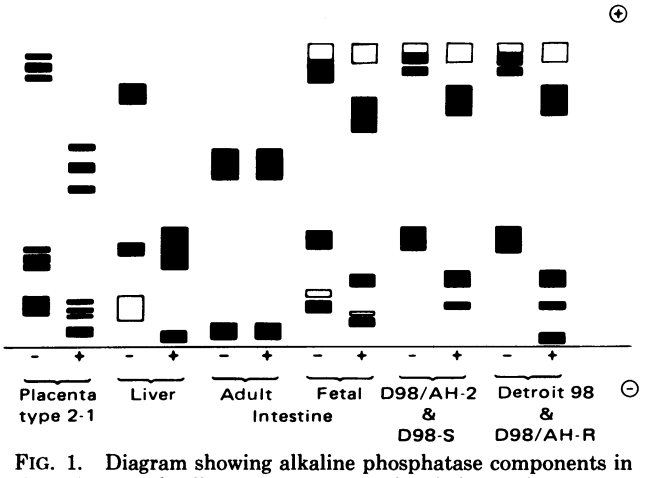

FIG. 1. Diagram showing alkaline phosphatase components in various tissue and cell extracts after starch gel electrophoresis. ■, Strongly staining band; □, weakly staining band. +, Treated with neuraminidase; −, not treated with neuraminidase.

Table 3. Percent alkaline phosphatase activity remaining in the presence of various inhibitors

Inhibitor	Alkaline phosphatase controls		D98/S	D98/AH-R	Detroit-98
	Liver	Intestinal			
L-Phenylalanine (2.5 mM)	92.30 ± 1.50	25.97 ± 0.75	24.98 ± 0.73	45.53 ± 1.20	55.38 ± 3.70
L-Homoarginine (10.0 mM)	20.53 ± 0.73	80.47 ± 1.08	82.43 ± 1.57	62.03 ± 1.43	50.23 ± 2.36
L-Leucine (5.0 mM)	72.91 ± 1.36	38.58 ± 0.94	39.32 ± 0.79	46.18 ± 1.39	53.73 ± 3.96
L-Leucylglycylglycine (5.0 mM)	61.66 ± 1.21	80.41 ± 1.09	82.58 ± 1.05	69.45 ± 1.15	67.43 ± 2.52
L-Phenylalanylglycylglycine (1.0 mM)	96.21 ± 1.00	80.11 ± 1.18	82.28 ± 1.32	78.45 ± 1.54	82.28 ± 0.56

Alkaline phosphatases of D98/S, D98/AH-R, and Detroit-98 were compared with liver and intestinal alkaline phosphatases. Means ± SEM are given.

Other Cell Lines Related to D98/AH-2. D98/AH-2 was originally derived in 1959 from a clonal colony of line D98/AG by selection with 8-azahypoxanthine (27). D98/AG itself was a clonal subline derived from line D98/S by selection with azaguanine. D98/S was originally obtained from a single cell clone derived from a population of Detroit-98 cells (27). Although it originated from the bone marrow of a healthy white male, at some stage it was probably contaminated and overgrown by HeLa because the present Detroit-98, D98/S, D98/AG, and D98/AH-2 are glucuse-6-phosphate dehydrogenase type A and also have other characteristics of HeLa (21–25, *). The line D98/AH-R was selected as an 8-azahypoxanthine-sensitive "revertant" from a D98/AH-2 culture. Thus, the relationship of these different cell lines can be represented as follows (27):

Detroit 98 → D98/S → D98/AG
→ D98/AH-2 → D98/AH-R.

Thermostability studies showed that essentially all the alkaline phosphatase activity in each of these lines was destroyed at 65°C after 60 min, which is evidence that they do not have significant amounts of placental alkaline phosphatase. The inhibition results are given in Table 3. D98/S showed the same inhibition profile as D98/AH-2 (Table 1) and also intestinal alkaline phosphatase. Detroit-98 and D98/AH-R, however, showed patterns of behavior with the different inhibitors that suggest they contain both intestinal and liver type alkaline phosphatases. The best discriminators of intestinal and liver alkaline phosphatases are L-phenylalanine and L-homoarginine. It is possible, therefore, by using the inhibition values for intestinal and for liver alkaline phosphatases with these two inhibitors, to obtain independent estimates of the relative amounts of the two forms of alkaline phosphatase presumed to be present in Detroit-98 and D98/AH-R. The estimates obtained with L-phenylalanine and L-homoarginine inhibitions are in good agreement (Table 4). They suggest that in Detroit-98 about 53% of the total activity is intestinal alkaline phosphatase and 47% is liver-type alkaline phosphatase and that in D98/AH-R about 70% of the activity is intestinal alkaline phosphatase and 30% is liver-type alkaline phosphatase.

The electrophoretic patterns obtained with Detroit-98, D98/S, and D98/AH-R alkaline phosphatases are shown in Fig. 1. The D98/S pattern is the same as that of D98/AH-2 and the patterns of Detroit-98 and D98/AH-R are very similar, except that after treatment with neuraminidase an additional slow-moving component is seen in Detroit-98 and D98/AH-R. This probably represents a form of liver/bone/kidney alkaline phosphatase.

DISCUSSION

The inhibition and thermostability studies clearly show that the alkaline phosphatase in D98/AH-2 is intestinal in type. This conclusion is supported by earlier experiments with an antiserum raised against pure placental alkaline phosphatase (20). Both D98/AH-2 and intestinal alkaline phosphatase reacted identically with the antiserum and there was partial immunologic identity with placental alkaline phosphatase.

The electrophoretic results suggest that D98/AH-2 alkaline phosphatase more closely resembles fetal intestinal alkaline phosphatase than adult intestinal alkaline phosphatase. The electrophoretic differences between fetal intestinal and adult intestinal alkaline phosphatase have not yet been explained. They may represent differences in posttranslational modification or may reflect the existence of separate loci coding for the adult and fetal forms of the enzyme. Whichever explanation is correct, it is reasonable to conclude that at least one intestinal alkaline phosphatase locus is being expressed in D98/AH-2 and that the loci coding for placental or for liver/bone/kidney alkaline phosphatases are not. The slight electrophoretic differences consistently observed between D98/AH-2 and fetal intestinal alkaline phosphatase may be explained by differences in posttranslational modifications or by mutations during the earlier life of the cell lines.

Many HeLa cell lines express placental alkaline phosphatase. This is clearly not the case in Detroit-98 or the various D98 lines derived from it. Because there is compelling evidence (21–25, *) that these two groups of cell lines have a common ancestry, it would appear probable that the expression of placental alkaline phosphatase was lost at some stage prior to the separation of Detroit-98. A, perhaps, less likely possibility is that placental alkaline phosphatase expression only appeared in the HeLa lineage subsequent to the separation of Detroit-98.

The apparent expression of liver-type alkaline phosphatase

Table 4. Estimated percentage of intestinal and liver alkaline phosphatases in Detroit-98 and D98/AH-R

Inhibitor	Detroit-98, estimated %		D98/AH-R, estimated %	
	Intestinal enzyme	Liver-type enzyme	Intestinal enzyme	Liver-type enzyme
L-Phenylalanine	56	44	71	29
L-Homoarginine	50	50	69	31

The estimates were obtained as follows: If the percentage of intestinal alkaline phosphatase is x and that of liver alkaline phosphatase is $(100 - x)$, and if the percent activity remaining in the presence of the particular inhibitor is K for the unknown and I and L, respectively, for the intestinal and liver standards then $x = 100\,(K - L)/(I - L)$.

along with intestinal alkaline phosphatase in Detroit-98 and also in D98/AH-R, which was originally derived from D98/AH-2, was an unexpected finding. Its interpretation will depend on whether in these cell lines all cells produce both of the enzyme forms or whether some cells are producing one form of the enzyme and other cells, the other. We hope to resolve this question by studying the alkaline phosphatase in single cell clones obtained from each of the lines.

The expression of a placental-like alkaline phosphatase in many HeLa sublines and in certain non-HeLa cell lines derived from various malignancies has its counterpart in the appearance of a placental-like alkaline phosphatase in a proportion of malignant tumors *in vivo* and presumably reflects a similar abnormality in gene regulation (16, 28–31). An alkaline phosphatase with properties similar to fetal intestinal alkaline phosphatase has also been reported in occasional tumors, particularly malignant heptomas (32–35), but also other malignancies (36). Presumably, the phenomenon observed in these tumors corresponds to that which we have found in D98/AH-2 and related cell lines.

We thank David Buck for the mycoplasma testing of the cell cultures and Vickie Hannig, Don Lai, and Denise Boccelli for excellent technical assistance. The work was supported by Grants CA-20296 from the National Cancer Institute, U.S. Department of Health, Education, & Welfare, VC-231 from the American Cancer Society, and PCM76-82997 from the National Science Foundation.

1. Mulivor, R. A., Plotkin, L. I. & Harris, H. (1978) *Ann. Hum. Genet.* **42**, 1–13.
2. Mulivor, R. A., Hannig, V. L. & Harris, H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3909–3912.
3. Fishman, W. H. & Sie, H.-G. (1971) *Enzymologia* **41**, 141–167.
4. Neale, F. C., Clubb, J. S., Hotchkis, D. & Posen, S. (1965) *J. Clin. Pathol.* **18**, 359–363.
5. Moss, D. W. (1975) *Enzyme* **20**, 20–34.
6. Whitby, L. G. & Moss, D. W. (1975) *Clin. Chim. Acta* **59**, 361–367.
7. Petit Clerc, C. (1976) *Clin. Chem.* **22**, 42–48.
8. Harris, H. & Hopkinson, D. A. (1976) *Handbook of Enzyme Electrophoresis in Human Genetics* (North-Holland, Amsterdam).
9. Sussman, H. H., Small, P. A., Jr. & Cotlove, E. (1968) *J. Biol. Chem.* **243**, 160–166.
10. Lehmann, F.-G. (1975) *Clin. Chim. Acta* **65**, 257–269.
11. Komoda, T. & Sakagishi, Y. (1976) *Biochim. Biophys. Acta* **445**, 645–660.
12. Khattab, M. & Pfleiderer, G. (1976) *Hoppe-Seyler's Physiol. Chem.* **357**, 377–391.
13. Vanneuville, J. F., Van Elsen, A. F. & Leroy, J. G. (1975) *Arch. Int. Physiol. Biochim.* **83**, 1017–1018.
14. Vanneuville, F. J., Van Elsen, A. F. & Leroy, J. G. (1977) *Biochem. Soc. Trans.* **5**, 1117.
15. Mulivor, R. A., Mennuti, M., Zackai, E. H. & Harris, H. (1978) *Am. J. Hum. Genet.* **30**, 271–282.
16. Fishman, W. H., Inglis, N. R., Green, S., Anstiss, C. L., Gosh, N. K., Reif, A. E., Rustigan, R., Krant, M. J. & Stolbach, L. L. (1968) *Nature (London)* **219**, 697–699.
17. Elson, N. A. & Cox, R. P. (1969) *Biochem. Genet.* **3**, 549–561.
18. Beckman, G., Beckman, L. & Lundgren, E. (1970) *Hum. Hered.* **20**, 182–186.
19. Tan, K. K. & Aw, S. E. (1971) *Biochim. Biophys. Acta* **235**, 119–127.
20. Benham, F. J., Povey, S. M. & Harris, H. (1978) *Somatic Cell Genet.* **4**, 13–25.
21. Gartler, S. M. (1967) *Natl. Cancer Inst. Monogr.* **26**, 167–195.
22. Gartler, S. M. (1968) *Nature (London)* **217**, 750–751.
23. Povey, S., Hopkinson, D. A., Harris, H. & Franks, L. M. (1976) *Nature (London)* **264**, 60–63.
24. Nelson-Rees, W. A. & Flandermeyer, R. R. (1976) *Science* **191**, 96–98.
25. Lavappa, K. S., Macy, M. L. & Shannon, J. E. (1976) *Nature (London)* **259**, 211–213.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
27. Szybalski, W., Hunter-Szybalski, E. & Ragni, G. (1962) *Natl. Cancer Inst. Monogr.* **7**, 75–88.
28. Stolbach, L. L., Krant, M. J. & Fishman, W. H. (1969) *N. Engl. J. Med.* **281**, 757–762.
29. Nathanson, L. & Fishman, W. H. (1971) *Cancer* **27**, 1388–1397.
30. Greene, P. J. & Sussman, H. H. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2936–2940.
31. Benham, F. J., Povey, S. M. & Harris, H. (1978) *Clin. Chim. Acta* **86**, 201–215.
32. Warnock, M. L. & Reisman, R. (1969) *Clin. Chim. Acta* **24**, 5–11.
33. Higashino, K., Ohtani, R., Kudo, S., Hashinotsume, M., Hada, T., Kang, K.-Y., Ohkochi, T., Takahashi, Y. & Yamamura, Y. (1975) *Ann. Intern. Med.* **83**, 74–78.
34. Suzuki, H., Iino, S., Endo, Y., Torii, M., Miki, K. & Oda, T. (1975) *Ann. N. Y. Acad. Sci.* **259**, 307–320.
35. Higashino, K., Otani, R., Kudo, S. & Yamamura, Y. (1977) *Clin. Chem.* **23**, 1615–1623.
36. Higashino, K., Kudo, S., Ohtani, R. & Yamamura, Y. (1976) *Gann* **67**, 909–911.