unlikely. Furthermore, we have demonstrated X-linkage of human steroid sulfatase by human-rodent somatic cell hybridization techniques (*20*). The second alternative is that there has been some strong selective pressure operative either in vivo or in vitro which favors the growth of cells with normal steroid sulfatase activity and inhibits the growth of sulfatase-deficient cells. In appropriate growth and mixing experiments, we have not observed such selection against steroid sulfatase-deficient cells in vitro. To rigorously exclude this possibility however, we have made use of the fact that subjects C and D are also heterozygous for G6PD deficiency of the Mediterranean type (*21*). As indicated in Fig. 2, when clones from these doubly heterozygous subjects were examined for G6PD activity as well as steroid sulfatase activity, clones with normal and deficient G6PD levels could be identified, but all clones had normal levels of steroid sulfatase activity. Thus, regardless of whether the X chromosome bearing the normal or the mutant G6PD allele was inactivated, steroid sulfatase was always expressed.

The data presented here provide evidence in a somatic cell system for non-Lyonization of an X-chromosome locus. It is of particular interest that this locus, which affects steroid sulfatase expression, is in relatively close proximity to another non-Lyonized locus, Xg^a. It should now be possible to utilize X-autosome translocations in somatic cell hybridization studies to localize cytologically the noninactivated region of the X chromosome which contains these two loci.

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References and Notes

1. S. M. Gartler and R. J. Andina, *Adv. Hum. Genet.* **7**, 99 (1976).
2. H. J. Muller, *Harvey Lect.* **43**, 1 (1950).
3. M. F. Lyon, *Nature (London)* **190**, 372 (1961).
4. R. G. Davidson, H. M. Nitowsky, B. Childs, *Proc. Natl. Acad. Sci. U.S.A.* **50**, 481 (1963).
5. B. R. Migeon, V. M. Der Kaloustian, W. L. Nyhan, W. J. Young, B. Childs, *Science* **160**, 425 (1968).
6. J. Salzman, R. De Mars, P. Benke, *Proc. Natl. Acad. Sci. U.S.A.* **60**, 545 (1968).
7. S. M. Gartler, S. Chen, P. J. Fialkow, E. R. Giblett, *Nature (London) New Biol.* **236**, 149 (1972).
8. B. F. Deys, K. H. Grzeschick, H. Grzeschick, E. R. Jaffé, M. Siniscalco, *Science* **175**, 1002 (1972).
9. B. R. Migeon, J. A. Sprenkle, I. Liebaers, J. F. Scott, E. F. Neufeld, *Am. J. Hum. Genet.* **29**, 448 (1977).
10. G. Romeo and B. R. Migeon, *Science* **170**, 180 (1970).
11. B. R. Migeon and F. Huijing, *Am. J. Hum. Genet.* **26**, 360 (1974).
12. W. J. Meyer, B. R. Migeon, C. J. Migeon, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1469 (1975).
13. C. J. Witkop, *Oral Surg. Oral Med. Oral Pathol.* **23**, 121 (1967); E. Passarge and E. Fries, *Nature (London) New Biol.* **245**, 58 (1973); I. A. Crimp and D. M. Danks, *J. Pediatr.* **78**, 466 (1971); J. L. Frias and D. W. Smith. *ibid.* **72**, 606 (1968); C. B. Kerr, R. S. Wells, K. E. Cooper, *J. Med. Genet.* **3**, 169 (1966); F. C. Ricciuti, T. D. Gelehrter, L. E. Rosenberg, *Am. J. Hum. Genet.* **28**, 332 (1976); M. R. Capobianchi and G. Romeo, *Experientia* **32**, 459 (1976).
14. J. Goldstein, J. F. Marks, S. M. Gartler, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1425 (1971).
15. J. Ducos, Y. Marty, R. Sanger, R. R. Race, *Lancet* **1971-II**, 219 (1971); P. J. Fialkow, *Am. J. Hum. Genet.* **22**, 460 (1970); ——, R. Lisker, E. R. Giblett, C. Zavala, *Nature (London)* **226**, 367 (1970).
16. M. A. Ferguson-Smith, *J. Med. Genet.* **2**, 142 (1965).
17. L. J. Shapiro, L. Cousins, A. L. Fluharty, R. L. Stevens, H. Kihara, *Pediatr. Res.* **11**, 894 (1977); L. J. Shapiro, R. Weiss, D. Webster, J. T. France, *Lancet* **1978-I**, 70 (1978); L. J. Shapiro, R. Weiss, M. M. Buxman, J. Vidgoff, R. L. Dimond, J. A. Roller, R. S. Wells, *ibid.* **1978-II**, 756 (1978).
18. C. B. Kerr, R. S. Wells, R. Sanger, *Lancet* **1964-II**, 1369 (1964); A. Adam, L. Ziprkowski, A. Feinstein, R. Sanger, R. R. Race, *ibid.* **1966-I**, 877 (1966); R. S. Wells, M. C. Jennings, R. Sanger, R. R. Race, *ibid.* **1966-II**, 494 (1966); A. Adam, L. Ziprkowski, A. Feinstein, R. Sanger, P. Tippett, J. Gavin, R. R. Race, *Ann. Hum. Genet.* **32**, 323 (1969); L. N. Went, W. P. deGroot, R. Sanger, P. Tippett, J. Gavin, *ibid.*, p. 333; G. Filippi, P. Meera Khan, *Am. J. Hum. Genet.* **20**, 564 (1968).
19. G. Romeo, A. Rinaldi, F. Urbano, G. Filippi, *Am. J. Hum. Genet.* **28**, 506 (1976).
20. T. Mohandas, R. Sparkes, L. J. Shapiro, unpublished results.
21. We thank M. Siniscalco, G. Filippi, and A. Rinaldi and their colleagues who identified these families in population studies of X-linked disorders in Sardinia. The G6PD phenotypes were established by histochemistry and quantitative assays of red blood cells [A. Rinaldi, G. Filippi, M. Siniscalco, *Am. J. Hum. Genet.* **28**, 496 (1976)].
22. Supported by NIH grant HD12178 from the National Institute of Child Health and Human Development, by grant 1-639 from the National Foundation-March of Dimes, and by the National Research Council of Italy.

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Separation of D and L Amino Acids by Liquid Chromatography: Use of Chiral Eluants

Abstract. *An aqueous eluant containing a chiral copper-proline complex effects the separation of underivatized amino acid enantiomers on an ion-exchange column. The stereoselectivity is ascribed to differences in stability of the diastereomeric amino acid–copper complexes formed in solution. A simple change in the chirality of the eluant reverses the order of the enantiomer elution. For detection and quantification of picomole amounts of amino acids, the eluant is monitored for fluorescence after reaction with* o*-phthalaldehyde, a reagent insensitive to proline but highly sensitive for amino acids containing a primary amino group.*

Chromatographic resolution of optical isomers (*1*) requires the introduction of an asymmetric environment either *intra*molecularly, by conversion to diastereomers, or *inter*molecularly, by the use of chiral stationary or mobile phases. In gas chromatography, excellent resolution of derivatized amino acids has been achieved with diastereomers (*2*), as well as with chiral stationary phases (*3*). Similarly in liquid chromatography, both these approaches have led to good separations of, for example, diastereomeric dipeptides (*4*) and helicenes on optically active supports (*5*).

In contrast, the effect of chiral *eluants* has not been extensively investigated (*6*). We now report a simple procedure for the separation of a number of α-amino acid enantiomers without the need for prior derivatization.

The method is based on the addition of a metal cation–amino acid complex to the eluant of a cation-exchange column. In the specific application reported, Cu^{2+}-proline complexes are dissolved (the molar ratio of Cu^{2+} to proline being 1/2) in a sodium acetate buffer. After the column is equilibrated (*7*), an amino acid sample is injected and is resolved into its enantiomers (Fig. 1). The chromatographic system in which the experiments were carried out had been developed (*8*) for the rapid, highly sensitive ion-exchange analysis of amino acids with the use of 5-μm bead resins. Separation was monitored by fluorometry (*9*) after the postcolumn reaction of the eluant with *o*-phthalaldehyde (*10*). To prevent precipitation of copper compounds by the *o*-phthalaldehyde solution, EDTA was added to the reagent (2.5 g/liter). *o*-Phthalaldehyde does interact with primary but not with secondary amines, so that proline does not interfere (nor does Cu^{2+}). Because of these circumstances the resolutions reported could be observed. The method is sensitive to picomole amounts.

Cysteic acid retention time was taken to represent the void volume of the column and the detection system (Table 1). The order of elution of the enantiomers (Fig. 1a and Table 1) with the chiral eluant is the reverse of that found by Rogozhin *et al.* (*11*) and Lefebvre *et al.* (*12*), who bonded a chiral proline-copper complex to the stationary support. This

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observation indicates that the stereoselectivity is due to interactions in the mobile phase rather than on the resin surface.

The aromatic amino acids show particularly efficient separation. With the tyrosine enantiomers, for example, the separation factor (r is the ratio of the adjusted retention times of the second peak over those of the first peak) is equal to 1.28 under the conditions described in Table 1. On a 3-cm column, complete resolution of tyrosine could be obtained in 5 minutes. At least some of the enantiomer pairs (Table 1) with separation factors of 1.00, indicating no resolution, can be separated using different operating parameters. For instance, D,L-asparagine, which has $r = 1.00$ under the conditions of Table 1, shows partial resolution at lower ionic strength and pH.

Temperature effects are evident. D,L-Valine gives only one peak at 25°C but baseline resolution at 75°C. Similar observations were made by Lefebvre *et al.* (*12*) with their chiral support. Some caution in the use of higher temperatures must, however, be exercised, because of possible racemization during chromatography. Indeed, L-serine showed a slight but measurable increase in the content of the D enantiomer when the column was operated at 90°C, as compared with 60°C.

Many more amino acids probably could be resolved efficiently by adjusting

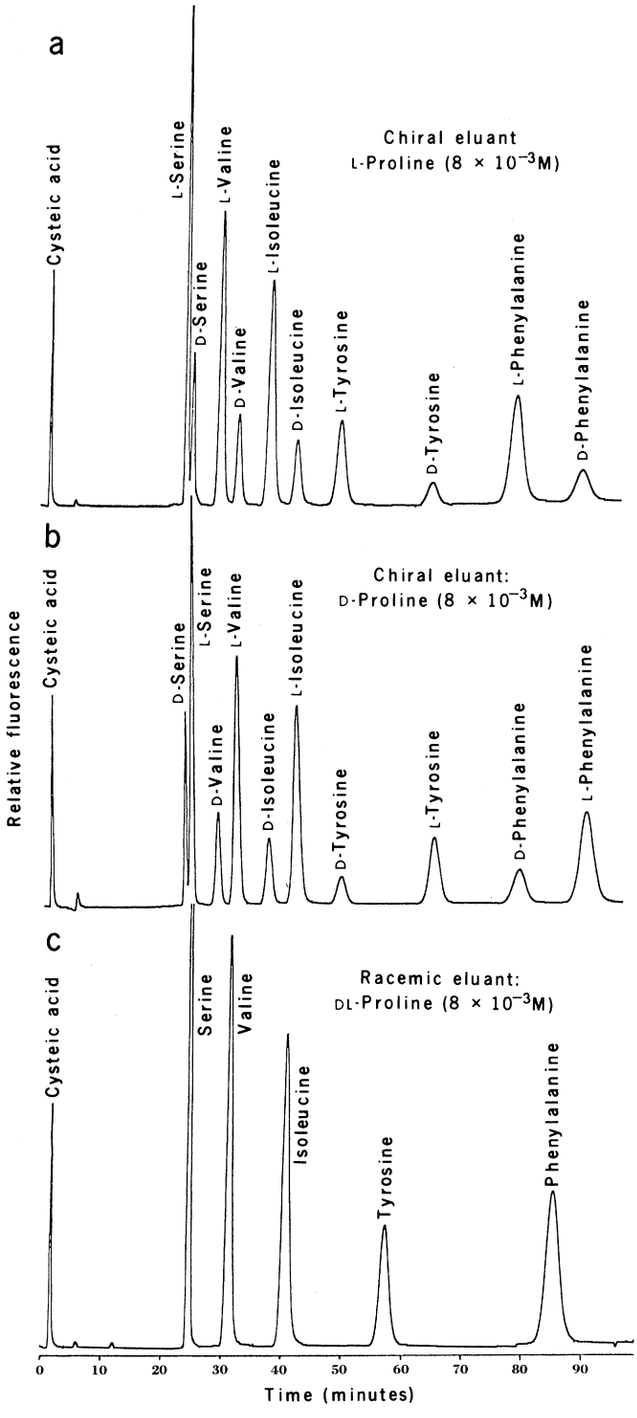

Fig. 1. Effect of the chirality of the eluant on the separation of D- and L-amino acid enantiomers by ligand-exchange chromatography. Identical portions of a mixture of five pairs of amino acid enantiomers, each consisting of 0.375 nmole of L form and 0.125 nmole D form, were injected in each run. Sodium acetate buffer (0.05N, pH 5.5) containing 4 × $10^{-3}M$ $CUSO_4$ and 8 × $10^{-3}M$ proline was used as eluant. The chirality of the proline ligand was as indicated. The column was equilibrated with each separate eluant for 15 minutes before the sample was injected. The column was 12 by 0.2 cm (inside diameter) packed with DC 4a resin. The eluant flow rate was 10 ml/hour, the reagent flow rate was 10 ml/hour, the column pressure was 200 bars, and the column temperature was 75°C. (a) L-Proline effected the separation of all five pairs of enantiomers with the L enantiomers eluting before the corresponding D enantiomers. (b) D-Proline reversed the order of elution. (c) With racemic proline no resolution occurred. The amino acids eluted halfway between the corresponding enantiomeric peaks in (a) and (b).

Table 1. Adjusted retention times (t'_R) and separation factors ($r = t'_{R(D)}/t'_{R(L)} = k'_D/k'_L$) for some amino acid enantiomers in ligand-exchange chromatography with a chiral eluant. Mobile phase: 0.1N sodium acetate with 8 × $10^{-3}M$ $CuSO_4 \cdot 5H_2O$ and 16 × $10^{-3}M$ L-proline. Column and operating details are as described in the Fig. 1 legend. Retention time for cysteic acid taken as column void volume.

Amino acid	Form	t'_R (min)	Separation factor (r)
(Cysteic acid)		(0)	
Aspartic acid	L	2.2	1.00
	D	2.2	
Glutamic acid	L	3.3	1.00
	D	3.3	
Allothreonine	L	10.0	1.14
	D	11.4	
Glutamine	L	12.9	1.00
	D	12.9	
Serine	L	12.9	1.04
	D	13.4	
Threonine	L	13.0	1.05
	D	13.7	
Asparagine	L	14.0	1.00
	D	14.0	
α-Amino-*n*-butyric acid	L	16.2	1.02
	D	16.6	
Valine	L	16.3	1.09
	D	17.7	
Alanine	L	17.2	1.00
	D	17.2	
Isoleucine	L	21.5	1.10
	D	23.7	
Norvaline	L	21.5	1.04
	D	22.3	
3,4-Dihydroxyphenylalanine	L	22.3	1.28
	D	28.5	
Alloisoleucine	L	23.5	1.09
	D	25.5	
Methionine	L	23.8	1.03
	D	24.6	
Leucine	L	28.3	1.01
	D	28.6	
Ethionine	L	29.0	1.04
	D	30.3	
Norleucine	L	30.8	1.05
	D	32.3	
Tyrosine	L	30.8	1.28
	D	39.4	
m-Tyrosine	L	34.1	1.21
	D	41.1	
o-Tyrosine	L	36.7	1.18
	D	43.2	
Phenylalanine	L	48.7	1.13
	D	55.0	
p-Fluorophenylalanine	L	64.0	1.18
	D	75.6	

the temperature, the pH, and the ionic strength. The basic amino acids such as lysine, histidine, and arginine, which do not emerge under the conditions described (Table 1), could possibly be eluted in a reasonable time at higher ionic strength or on a shorter column (or both).

Additional options for obtaining desired resolutions are the use of chiral ligands differing from proline, including other amino acids, their derivatives, or other classes of compounds. Changing the nature of the cation offers further possibilities. For instance, hydroxyproline-Cu^{2+} complexes show stereoselectivity as good as or better than that of proline (*13*).

When an L-proline ligand is used, the L enantiomer of the amino acid resolved is eluted before the corresponding D isomer (Fig. 1a). This order is reversed when a D-proline–copper complex is dissolved in the eluant (Fig. 1b), and there is no resolution with a racemic proline-copper complex in the eluant (Fig. 1c). Switching chirality of the eluant offers a useful method for distinguishing nonchiral artifacts from true enantiomers in a sample and requires only a few minutes for equilibrating the column. Retention times can be accurately reproduced (Fig. 1).

The stereoselectivity is ascribed to differences in the stability constants of diastereomeric species such as the L-proline–Cu^{2+} complex with an L-amino acid and the L-proline-Cu^{2+} complex with a D-amino acid in aqueous solution. Qualitative and quantitative differences of stability constants have been reported (*14*), and it is surprising that resolutions of amino acids similar to those described here have not been reported before (*15*).

Our results indicate the feasibility of a simple automated procedure for quantitative protein amino acid analysis with simultaneous determination of enantiomeric composition. The method will be useful, for example, in checking synthetic and naturally occurring biologically active peptides for the presence of D-amino acid components, for screening physiological fluids for D-amino acids, and for ascertaining amounts of D-amino acid enantiomers in progressively older fossils (*16*), in certain living tissues such as tooth dentin and enamel, and in lens proteins of cataract patients (*17*).

The presence of naturally occurring chiral ligands coordinated to metals and their role in the geochemical transport and distribution of enantiomers and trace elements is a question of interest arising from our study.

Our experiments establish the feasibility of resolution of underivatized α-amino acids on an ion-exchange column with a chiral aqueous eluant. LePage *et al.* (*18*) achieved resolution of *dansyl* amino acids by reverse phase chromatography and an eluant containing a zinc-chiral ligand complex. These two studies and those referred to earlier (*6*) illustrate the additional refining in separation power that can be achieved by modifying the eluant composition (*19*).

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References and Notes

1. The D- and L-amino acid enantiomers are mirror-image isomers of the same amino acid with identical physical properties, except that they rotate the plane of polarized light in opposite directions. Enantiomers also have identical chemical properties except toward chiral (optically active) reagents.
2. R. Charles, G. Fischer, E. Gil-Av, *Isr. J. Chem.* **1**, 234 (1963); G. E. Pollock, V. I. Oyama, R. D. Johnson, *J. Gas Chromatogr.* **3**, 174 (1965).
3. *N*-Lauroyl-L-valine t-butylamide is an example of a useful chiral stationary phase. B. Feibush, *Chem. Commun.* (1971), p. 544; R. Charles, U. Beitler, B. Feibush, E. Gil-Av, *J. Chromatogr.* **112**, 121 (1975). See also H. Frank, G. J. Nicholson, E. Bayer, *J. Chromatogr. Sci.* **15**, 174 (1977).
4. J. M. Manning and S. Moore, *J. Biol. Chem.* **243**, 5591 (1968).
5. F. Mikes, G. Boshart, E. Gil-Av, *J. Chromatogr.* **122**, 205 (1976); H. Numan, R. Helder, H. Wynberg, *Recueil* **95**, 211 (1976).
6. W. H. Pirkle and D. L. Sikkenga, *J. Chromatogr.* **123**, 400 (1976); H. Nakazawi and H. Yoneda, *ibid.* **160**, 89 (1978) and references therein.
7. Equilibration of the ion-exchange column to the copper form requires several hours unless the resin is converted to the copper form before the column is packed. Once equilibrated with copper, the various eluants can be interchanged with minimal reequilibration so long as the copper-ion concentration is not altered.
8. P. E. Hare, *Methods Enzymol.* **47**, 3 (1977). Dionex Corporation (1228 Titan Way, Sunnyvale, Calif. 94086) offers an amino acid analyzer kit patterned after the system described above. They also supplied the DC 4a resin.
9. Both the Fluoro-monitor (Aminco catalog No. J4-7461) and a prototype of an auxiliary Fluoromonitor (Aminco catalog No. J4-7502) were used.
10. M. Roth, *Anal. Chem.* **43**, 880 (1971); J. R. Benson and P. E. Hare, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 619 (1975).
11. S. Rogozhin and V. Davankov, *J. Chem. Soc. D* (1971), p. 490, and references therein.
12. B. L. Lefebvre, R. Audebert, C. Quivoron, *Isr. J. Chem.* **15**, 69 (1977) and references therein.
13. P. E. Hare and E. Gil-Av, unpublished observations.
14. For example, B. L. Leach and R. J. Angelici, *J. Am. Chem. Soc.* **91**, 6297 (1969).
15. The experiments reported here were planned after our discussion at the Carnegie Institution of Washington Conference on "Advances in the Biogeochemistry of Amino Acids," Airlie House, Warrenton, Va., 29 October to 1 November 1978.
16. P. E. Hare and P. H. Abelson, *Geol. Soc. Am. Spec. Pap.* **115**, 91 (1967); *Carnegie Inst. Washington Yearb.* **66**, 526 (1968); P. E. Hare, in *Organic Geochemistry, Methods and Results*, G. Eglinton and M. T. J. Murphy, Eds. (Springer-Verlag, New York, 1969), pp. 438–463.
17. P. M. Helfman and J. L. Bada, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2891 (1975); P. M. Helfman and J. L. Bada, *Nature (London)* **262**, 279 (1976); P. M. Masters, J. L. Bada, J. S. Zigler, *ibid.* **268**, 71 (1977).
18. J. N. LePage, W. Lindner, G. Davies, D. E. Seitz, B. L. Karger, *Anal. Chem.* **51**, 433 (1979).
19. B. L. Karger and R. W. Giese, *ibid.* **50**, 1048A (1978).

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Dendritic Mechanisms Underlying Penicillin-Induced Epileptiform Activity

Abstract. *The action of penicillin on synaptically evoked dendritic activity was examined with the use of hippocampal slice preparations. Orthodromic activation of CA1 pyramidal neurons produced an excitatory-inhibitory postsynaptic potential sequence recorded intracellularly in the dendrites. Treatment with penicillin resulted in the appearance of spontaneous and synaptically evoked multipeaked field potentials and associated depolarization shifts and spike burst generation in CA1 cells. Intracellular recordings revealed that penicillin produced no detectable change in passive membrane properties of the postsynaptic dendrites. However, the inhibitory postsynaptic potential was suppressed by penicillin, resulting in the release of intrinsic dendritic burst firing during synaptic activation. These findings emphasize the role of normal patterns of dendritic burst generation in the production of intense neuronal discharge during penicillin-induced epileptiform activities.*

Application of the convulsant drug penicillin to mammalian cortex leads to the development of interictal epileptiform discharges recorded in the electrocorticogram. Intracellular recordings obtained from neurons during the genesis of such events typically show high-amplitude (20 to 30 mV) prolonged (50 to 100 msec) membrane depolarization shifts (DS's) with overriding trains of action potentials. The mechanism underlying the generation of these DS's is an important issue directly relevant to our understanding of the basic mechanisms of epilepsy. Two hypotheses have been proposed (*1*). One suggests that DS's that give rise to burst firing are summated excitatory postsynaptic potentials (EPSP's) resulting from actions of penicillin which increase excitatory synaptic transmission (*2*) or decrease inhibitory synaptic events (*3*). A second hypothesis

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