Abstract. Familial hyperproinsulinemia, a hereditary syndrome in which individuals secrete high amounts of 9,000-mol wt proinsulin-like material, has been identified in two unrelated cohorts. Separate analysis of the material from each of the two cohorts had suggested that the proinsulin-like peptide was a conversion intermediate in which the C-peptide remained attached to the insulin B-chain in one case, whereas it was a conversion intermediate in which the C-peptide remained attached to the insulin A-chain in the other. To reinvestigate this apparent discrepancy, we have now used chemical, biochemical, immunochemical, and physical techniques to compare in parallel the structures of the immunoaffinity chromatography-purified, proinsulin-like peptides isolated from the serum of members of both families. Our results show that affected individuals in both cohorts secrete two-chained intermediates of proinsulin conversion in which the COOH-terminus of the C-peptide is extended by the insulin A-chain and from which the insulin B-chain is released by oxidative sulfotylisis. Analysis of the conversion intermediates by reverse-phase high-performance liquid chromatography using two different buffer systems showed that the proinsulin-related peptides from both families elute at a single position very near that of the normal intermediate des-Arg$^{31}$,Arg$^{32}$-proinsulin. Further, treatment of these peptides with acetic anhydride prevented trypsin-catalyzed cleavage of the C-peptide from the insulin A-chain, a result demonstrating the presence of Lys$^{64}$ and the absence of Arg$^{65}$ in both abnormal forms. We conclude that individuals from both cohorts with familial hyperproinsulinemia secrete very similar or identical intermediates of proinsulin conversion in which the C-peptide remains attached to the insulin A-chain and in which Arg$^{65}$ has been replaced by another amino acid residue.

Introduction

Several insulin gene mutations resulting in the biosynthesis and secretion of abnormal gene-related products are now recognized to be associated with human diabetes. Three unrelated patients with insulin gene mutations are known to secrete unique, abnormal insulins with decreased biological activity (1-5); in two of these individuals, the sites of genetic mutation and the identities of the corresponding amino acid replacements have been determined by techniques involving both peptide chemistry and recombinant DNA analysis (4-7). Two additional patients are known to secrete abnormal proinsulin-like peptides rather than insulin-like peptides (8-10). Patients in the latter group have been classified as having a syndrome called familial hyperproinsulinemia, and inherit the gene defect in an autosomal dominant pattern (8). Although such individuals exhibit serum levels of total immunoreactive insulin as high as 100 μU/ml, 85-90% of their serum immunoreactive insulin has a molecular weight indistinguishable from that of proinsulin; in the absence of complicating factors, most subjects with familial hyperproinsulinemia show normal glucose tolerance.

Structural analysis of the immunoreactive material present in serum from both cohorts with familial hyperproinsulinemia has suggested that all affected patients secrete intermediates of proinsulin conversion rather than intact proinsulin, and that the intermediates arise from incomplete processing of an abnormal proinsulin in which an amino acid replacement has occurred at one of the usual paired dibasic amino acid conversion sites (9, 10). Using chemical and immunochemical methods, we identified the proinsulin-related material from the one cohort with familial hyperproinsulinemia (called cohort 2) as a two-chained intermediate of proinsulin conversion in which the C-peptide remained attached to the insulin A-chain (a type II intermediate) (10); preliminary studies had suggested that the
proinsulin-related material from the other cohort (called cohort 1) was a conversion intermediate in which the C-peptide remained attached to the insulin B-chain (a type I intermediate) (9). Types I and II proinsulin conversion intermediates are formed during the normal processing of proinsulin within the pancreatic B cell and represent, in the case of man, human des-Lys46,Arg55-proinsulin and human des-Arg31,Arg32-proinsulin, respectively. As the physiological consequences of secreting a type I proinsulin conversion intermediate are different from those of secreting a type II intermediate (11, 12), and as the structural identification of these intermediates bears on an understanding of mechanisms for the processing of the hormone precursor, we undertook a reexamination of the 9,000-mol wt material isolated from the serum of individuals of cohort 1. In correction of the original proposal (9), we have now identified the proinsulin-related material from these individuals as a two-chained intermediate of proinsulin conversion in which the C-peptide remains attached to the insulin A-chain and in which Arg55 is replaced by another amino acid. Further, the intermediates from both cohorts elute from reverse-phase columns at the same position during high-performance liquid chromatography (HPLC) analysis. We conclude that members of both cohorts with familial hyperproinsulinemia secrete type II intermediates of proinsulin conversion that arise from similar or identical insulin gene mutations.

Methods

Isolation and measurement of serum proinsulin-like material. In most cases, insulin antibodies were purifed from 5 ml of guinea pig anti-insulin serum which was diluted with two parts of 0.1 M boric acid brought to pH 8 with NaOH, by passing the solution through a column (1 x 10 cm) of insulin-Sepharose (prepared from cyanogen bromide-activated Sepharose 4B and porcine insulin, 2 mg/ml, according to the manufacturer’s instructions) at 22°C using a flow rate of 20 ml/h. The column was washed first with 50 ml of the buffer, then with 20 ml of water. The antibodies were eluted by 20% acetic acid, concentrated by lyophilization, and coupled to Sepharose 4B as before to obtain the immunofinity chromatography medium used for purifying serum insulin. Patient serum (10–40 ml) was diluted with two volumes of the borate buffer described above; the diluted serum was filtered through Whatman No. 1 paper (Whatman Laboratory Products Inc., Clifton, NJ) to remove insoluble material, and the filtrate was applied to a column (1 x 15 cm) of anti-insulin IgG-Sepharose 4B at 4°C using a flow rate of 4 ml/h. The column was washed sequentially with 50 ml of the borate buffer containing 0.5% (wt/vol) bovine serum albumin, 30 ml of the buffer alone, and 20 ml of water. The serum insulin-related material was eluted with 20% acetic acid containing 10 μg/ml of albumin; 1-ml fractions were collected and assayed for immunoreactive insulin by radioimmunoassay. Fractions containing insulin plus proinsulin were concentrated and gel filtered on a column (1.5 x 60 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, CA) equilibrated with 20% acetic acid containing 10 μg/ml of albumin. Aliquots of the fractions (1 ml) were again assayed for immunoreactive insulin and fractions containing the 9,000- and 6,000-mol wt insulin-immunoreactive components were separately pooled for storage at −20°C and further study. Methods for the radioimmunoassay of insulin using antisera GP-1 (13), for the radioimmunoassay of C-peptide using antisera M1230 (14), and for the radioimmunoassay for S-sulfo-insulin B-chain (15) have been described. All serum samples were obtained from fasted individuals; levels of total immunoreactive insulin varied from 70 to 100 μU/ml among the different samples.

Enzymatic and chemical modification of proinsulin and proinsulin-like material. Conditions have been described for the digestion of immunofinity-puriﬁed serum proinsulin-like material by tosylphenylalanne chloride, sodium acetate, triethylamine, and acetonitrile to obtain insulin C-peptide (both authentic and immunoreactive human C-peptide) by comparison during HPLC with authentic standards (prepared by carboxypeptidase B treatment of split proinsulins) provided by Dr. Bruce Frank, Eli Lilly & Co. Because authentic standards were available in only very low amounts, the enzyme-digested peptide mixture was used as the routine HPLC standard.

Peptide separations. Gel filtration of purified serum proinsulin-like material both before and after oxidative sulfitolysis proceeded on columns (0.9 x 90 cm) of Bio-Gel P-10 equilibrated with a buffer containing 0.1 M Tris, 0.05 M NaCl, 0.2 mg/ml sodium azide, and 5 μg/ml of bovine serum albumin, all brought to pH 7.6 with HCl; 1.2-ml fractions were collected and the flow rate was 6 ml/h. Aliquots of each fraction were assayed for immunoreactive insulin, immunoreactive S-sulfo-insulin B-chain, and immunoreactive human C-peptide (both in the native state and after digestion with trypsin and carboxypeptidase B) as appropriate. Samples for polyacrylamide gel electrophoresis, and by comparison during HPLC with authentic standards (prepared by carboxypeptidase B treatment of split proinsulins) provided by Dr. Bruce Frank, Eli Lilly & Co. For radioimunoassay of C-peptide immunoreactivity (10). HPLC separations of immunopuriﬁed proinsulin-like material proceeded on an Altex C-18 ultrashere ion-pair column (0.46 x 25 cm) using a Perkin-Elmer Series 4 liquid chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped with an ISS-100 automatic sampler. The flow rate was 1 ml/min and 0.4-ml fractions of column effluent were collected in all cases; fractions were dried under vacuum and the residues were dissolved in buffer for assay of both insulin and C-peptide immunoreactivity. Two aqueous HPLC buffer systems were used. The first contained 0.1 M phosphoric acid, 0.02 M triethylamine, and 0.05 M NaClO4, all adjusted to pH 3 with NaOH; the second contained 0.05 M phosphoric acid, 0.01 M hexane sulfonic acid, and 0.10 M NaClO4, all adjusted to pH 3 with NaOH. The organic solvent was acetonitrile in both cases; see figure legends for details.
Results

Our examination of the serum proinsulin-like material from subjects of cohort 1 began with an assessment of the immunoreactive C-peptide and B-chain moieties of the 9,000-mol wt peptide. The gel filtration profiles of Fig. 1a show that the purified peptide reacted on a molar basis only about one-fourth as well with C-peptide antibodies as it did with insulin antibodies. Treatment of the column fractions with trypsin and carboxypeptidase B (a procedure that releases C-peptide from proinsulin and proinsulin intermediates by cleavage at paired dibasic amino acid conversion sites and by removal of COOH-terminal basic residues) resulted in a major increase in C-peptide immunoreactivity. As treatment of the material with trypsin or carboxypeptidase B alone did not significantly enhance C-peptide immunoreactivity (not shown, see reference 10), and as our C-peptide antiserum reacts best with C-peptide having a free COOH-terminus (17), the result shows that the C-peptide bears a COOH-terminal extension in the 9,000-mol wt peptide. Treatment of the intact proinsulin-like material by oxidative sulfotolysis (a procedure that breaks disulfide bonds and releases disulfide bond-linked peptide chains) before gel filtration resulted in a large peak of immunoreactive B-chain and a small peak of high molecular weight C-peptide-immunoreactive material (Fig. 1b); as before, treatment of the C-peptide-containing material with trypsin and carboxypeptidase B greatly enhanced its immunoreactivity. The combined results of Fig. 1, a and b, show that the proinsulin-like material in the serum of members of cohort 1, like that present in the serum of members of cohort 2 (10), consists of a two-chained intermediate of proinsulin conversion in which the C-peptide is extended from its COOH-terminus by the insulin A-chain and in which the extended A-chain is disulfide bonded to native insulin B-chain (a type II proinsulin intermediate).

To compare the proinsulin conversion intermediates from members of cohorts 1 and 2 in greater detail, we developed reverse-phase HPLC systems to separate types I and II proinsulin intermediates both from each other and from insulin and proinsulin. HPLC profiles resulting from the use of triethylammonium phosphate buffers are illustrated in Fig. 2. Panel a shows the resolution of human insulin, the two human conversion intermediates, and human proinsulin on the C-18 column. Notably, the ratios of immunoreactive C-peptide to immunoreactive insulin measured for the type II conversion intermediate and for proinsulin (two compounds in which the COOH-terminus of the C-peptide is blocked) were about 0.25:1, whereas that ratio for the type I conversion intermediate (a compound containing a free C-peptide COOH-terminus) was about 1:1. Panels b and c of Fig. 2 show HPLC elution profiles of total insulin-immunoreactive material isolated from the serum of members of cohorts 1 and 2, respectively. Both profiles show low amounts of insulin and much higher amounts of a peptide that reacted with both insulin and C-peptide antibodies and that eluted at the position of the type II proinsulin conversion intermediate. Further, the low ratio of C-peptide to insulin immunoreactivity in this material (~0.25) was typical of that shown by a type II intermediate containing a blocked C-peptide COOH-terminus. Results from a second HPLC system, for which the anion pair reagent triethylamine was replaced by hexane sulfonic acid (a cation pair reagent), are shown in Fig. 3. Resolution of insulin, types I and II conversion intermediates, and proinsulin was complete (Fig. 3a); again, the major fractions of serum proinsulin-like material from subjects of cohorts 1 and 2 eluted at the position (and with the selective immunoreactivity) of a type II intermediate of proinsulin conversion (Fig. 3, c and d).

Analysis of the proinsulin intermediates of cohorts 1 and 2 by polyacrylamide gel electrophoresis provided further information on their structural similarities. Fig. 4 shows that (a) the proinsulin intermediates from members of both cohorts migrate further than native proinsulin during polyacrylamide gel electrophoresis at pH 8.7 (a finding consistent with the loss of dibasic amino acids during the formation of the intermediates), and (b) in both cases, the immunoreactive C-peptide cleaved from the intermediates by digestion with trypsin and carboxypeptidase B has the character of normal human C-peptide. We reasoned, as we had in our studies on the intermediate from cohort 2 (10), that the most likely sites for structural change in the intermediate from cohort 1 were those associated with the normal, paired dibasic amino acid conversion site Lys44-Arg65. Studies based on the susceptibilities of the intermediates to acetylation (a procedure that derivatizes amino groups and removes their positive charges) permitted an identification of the locus of amino
Figure 2. Reverse-phase HPLC elution profiles of immunopurified material isolated from the serum of subjects from cohorts 1 and 2 using triethylamine-containing buffer. Panel a shows the standard profile obtained from chromatography of a sample of human proinsulin digested briefly with a mixture of trypsin and carboxypeptidase B. The elution positions of insulin, the type II intermediate of conversion (Int II, in which the C-peptide is attached to the insulin A-chain), the type I intermediate of conversion (Int I, in which the C-peptide is attached to the insulin B-chain), and proinsulin (Proins) were confirmed by use of standard preparations provided by Dr. Bruce Frank of Eli Lilly & Co. Left and right arrowheads at the top of the figure show the elution positions of the type II and type I proinsulin standards, respectively. Panels b and c show profiles obtained after HPLC chromatography of samples of immunopurified material from the serum of subjects of cohorts 1 and 2, respectively; these samples were not gel filtered before HPLC analysis. Aliquots of collected fractions were analyzed for immunoreactive insulin (●) and C-peptide (○). In all cases, the reverse-phase column was sequentially eluted with an isocratic solution of 30.5% acetonitrile in triethylamine-containing buffer (20 min), a linear gradient from 30.5 to 32.9% acetonitrile in the buffer (10 min), an isocratic solution of 32.9% acetonitrile in the buffer, and a linear gradient from 32.9 to 55% acetonitrile in the buffer (to remove albumin absorbed to the column, not shown in the figure); the column was equilibrated with the starting isocratic buffer solution for 60 min before the sample was applied.

As shown in Fig. 4a, treatment of normal human proinsulin with acetic anhydride followed by digestion by trypsin and carboxypeptidase B resulted in a peptide with the electrophoretic mobility of native C-peptide, but with the low C-peptide immunoreactivity characteristic of a C-peptide with a blocked COOH-terminus; these properties are consistent with those expected for the product ε-N-acetyl-C-peptideyllysine. On the other hand, treatment of the type II proinsulin intermediates from cohorts 1 and 2 with acetic anhydride before enzymatic digestion resulted in peptides that migrated more slowly than native C-peptide and again showed the low immunoreactivity expected for COOH-terminally extended C-peptide forms (Fig. 4, b and c). The susceptibilities of proinsulin intermediates from cohorts 1 and 2 to acetylation demonstrated that Lys83 was present in both cases; the failure of trypsin and carboxypeptidase B to cleave the acetylated intermediates to ε-N-acetyl-C-peptideyllysine demonstrated that Arg83 was absent from both abnormal type II intermediate forms. Further, the proinsulin intermediates from cohorts 1 and 2 appeared to be retained slightly longer than normal des-Arg31,Arg32-proinsulin during reverse-phase HPLC.
Figure 4. Polyacrylamide gel electrophoresis of serum proinsulin-like material from subjects of cohorts 1 and 2. Panels a, b, and c illustrate results obtained for normal human proinsulin, the material from subjects of cohort 1, and the material from subjects of cohort 2, respectively. In all cases, the tube gels were sectioned after electrophoresis and the material eluted from each slice was assayed for C-peptide immunoreactivity. Symbols are identified as follows: a, electrophoresis of the native peptide; b, electrophoresis of the peptide subjected to acetylation (to block amino groups) and then sequentially digested by trypsin and carboxypeptidase B; c, electrophoresis of the peptide digested by trypsin and carboxypeptidase B without prior acetylation. The arrow in panel a shows the position taken by native human C-peptide.

in hexane sulfonate-containing buffer (Fig. 3). This result is consistent with both intermediates containing a single locus of amino acid substitution; however, replacement of a single amino acid in a peptide containing 84 residues would not necessarily result in a modified form with greatly altered hydrophobicity or reverse-phase HPLC mobility.

Discussion

Although previous studies had suggested that the 9,000-mol wt peptides identified in familial hyperproinsulinemia cohorts 1 and 2 were type I and type II intermediates of proinsulin conversion, respectively (9, 10), our results now show that both peptides are type II intermediates; that is, both peptides arise from cleavage of proinsulin at the paired dibasic amino acid residues joining the insulin B-chain to the C-peptide, and both peptides are related to the normal conversion intermediate des-Arg\(^{31}\),Arg\(^{32}\)-proinsulin (11, 18). (See Fig. 5, upper left and upper right, for a comparison of the structures of type I and type II proinsulin conversion intermediates.) In fact, immunochromical, chemical, electrophoretic, and chromatographic methods show that the type II proinsulin conversion intermediates from cohorts 1 and 2 are indistinguishable, and that both intermediates contain amino acid substitutions replacing Arg\(^{35}\). As illustrated in Fig. 5, lower, replacement of Arg\(^{65}\) by another amino acid would alter the paired dibasic amino acid sequence normally serving as the site for conversion of the type II intermediate to insulin plus C-peptide. Although our methods and results do not exclude the possibility that Arg\(^{65}\) has been replaced by lysine (a replacement resulting in the less commonly observed Lys-Lys prohormone conversion site, reference 19), it is probable that Arg\(^{65}\) has been replaced by a nonbasic amino acid residue: a single nucleotide change in the human insulin gene sequence coding for Arg\(^{65}\) (CGT, reference 20) could result in replacements involving only leucine, serine, glycine, histidine, proline, or cysteine. As proline and cysteine substitutions would result, respectively, in intermediates not susceptible to cleavage by trypsin and in intermediates not disposed to correct disulfide bond formation, it is probable that the abnormal conversion intermediates from both cohorts arise from leucine, serine glycine, or histidine for arginine replacements at position 65 of human proinsulin.

The occurrence of 6,000-mol wt insulin as well as of the type II conversion intermediate in the serum of individuals with familial hyperproinsulinemia (in the approximate molar ratio 1:6) most likely arises from the codominant expression of both normal and abnormal insulin gene alleles. Loss of Arg\(^{65}\) in the expressed product of the abnormal allele apparently yields a proinsulin molecule that is not subject to normal enzymatic processing at the modified conversion site. The secretion of type II conversion intermediates (rather than abnormal proinsulins) in members of cohorts 1 and 2 demonstrates that the endogenous converting endopeptidase is present in both cases. Our results thus emphasize the validity of earlier proposals that suggested the importance of paired dibasic amino acid residues in directing the processing of both proinsulin (10) and proalbumin (21, 22).

It has not yet been determined with assurance whether the genetic defect in an additional and recently described cohort with familial hyperproinsulinemia (cohort 3) (23) arises from a mutation in the insulin gene or from an alteration in the converting endopeptidase.

Figure 5. Structures of normal and abnormal proinsulin conversion intermediates. The amino acid sequences and diagnostic structures of normal type I and type II human proinsulin conversion intermediates are shown at the bottom of this figure as an illustration of the type I intermediate and Lys-Arg in the case of a type II intermediate) are illustrated. The lower portion of the figure illustrates in greater detail amino acid sequences surrounding the potential conversion site in a type II intermediate. The COOH-terminal three residues of the C-peptide, the dibasic amino acid pair at the conversion site, and the NH\(_2\)-terminal three residues of the insulin A-chain are illustrated using the three letter amino acid code. The amino acid that replaces Arg\(^{65}\) in the abnormal type II intermediate from cohorts 1 and 2 has not yet been identified and is indicated as Xxx.
As subjects with familial hyperproinsulinemia are either eu-
glycemic (8, 9) or mildly diabetic (10), it is not surprising that
such individuals are found to secrete type II rather than type I
proinsulin conversion intermediates. The type I intermediate
(Fig. 5, upper left) has a biological activity nearly as high as
that of insulin itself (11, 12); if the 9,000-mol wt material present
in the serum of these familial hyperproinsulinemia patients were
to have been a type I intermediate, the patients would probably
have presented with symptoms of hypoglycemia. Indeed, the
type II proinsulin intermediate (Fig. 5, upper right) has low
biological activity (11, 12), a finding consistent with its slow
metabolic clearance, with its accumulation in plasma, and with
clinical findings associated with familial hyperproinsulinemia
subjects. Notwithstanding the low insulin/proinsulin ratio ob-
served in these patients (~0.15:1 compared with the normal
ratio of ~5:1), the concentration of serum insulin in affected
individuals (10–20 μU/ml) is actually sufficient to place them
within the normal range for fasting insulin levels. It is thus clear
that processes as diverse as expression of normal and abnormal
genetic alleles, processing of normal and abnormal hormone
precursors, and clearance of high and low activity gene products
all have important roles in determining the physiological and
biochemical findings associated with insulin gene mutations
in man.

Acknowledgments

The authors thank Dr. B. Frank for providing standards of normal
human proinsulin conversion intermediates, Dr. K. Gabbay and D. Y.
Kanazawa for providing serum samples, Petra Blix for technical assis-
tance, and Arlene Timosiek for assistance in preparing the manuscipt.

This work was supported by grants AM 18347, AM 29866, AM 13941, and AM 20595 from the National Institutes of Health.

References

1. Tager, H., B. Given, D. Baldwin, M. Mako, J. Markese, A. Ruben-
A structurally abnormal insulin causing human diabetes. Nature (Lond.)
281:122–125.
2. Given, B. D., M. E. Mako, H. S. Tager, D. Baldwin, J. Markese,
A. H. Rubenstein, J. Olefsky, M. Kobayashi, O. Koltermann, and R.
Poucher. 1980. Diabetes due to secretion of an abnormal insulin. N.
3. Haneda, M., R. Bergenstal, G. Friedenberg, W. Wishner, P. Blix,
S. Provon, K. Polonsky, and A. H. Rubenstein. 1982. Familial diabetes
associated with a possible structural defect in circulating insulin. Diabetes
31 (Suppl. 2):13.
4. Shoelsom, S., M. Haneda, P. Blix, A. Nanjo, T. Sanke, K. Inouye,
D. Steiner, A. Rubenstein, and H. Tager. 1983. Three mutant insulins
5. Shoelosom, S., M. Fickova, H. Haneda, A. Nahum, G. Musso,
a mutant human insulin predicted to contain a serine for phenylalanine
1983. Identification of a point mutation giving rise to a structurally
abnormal insulin (Insulin Chicago). Diabetes. 32:872–875.
7. Haneda, M., S. J. Chan, S. C. M. Kwok, A. H. Rubenstein, and
D. F. Steiner. 1983. Studies on mutant human insulin genes: identifica-
Acad. Sci. USA. 80:6366–6370.
8. Gabbay, K. H., K. DeLuca, J. N. Fisher, Jr., M. E. Mako, and
9. Gabbay, K. H., R. M. Bergenstal, J. Wolf, M. E. Mako, and
A. H. Rubenstein. 1979. Familial hyperproinsulinemia: partial charac-
Sci. USA. 76:2882–2885.
10. Robbins, D. C., P. M. Blix, A. H. Rubenstein, Y. Kanazawa,
studies on porcine proinsulin and related polypeptides. In Diabetes.
Amsterdam. 292–305.
and related polypeptides in the fat tissue. J. Biol. Chem. 248:3753–
3761.
1979. Insulin, proinsulin, and C-peptide. In Methods of Hormone Ra-
dioimmunoassay. B. M. Jaffe and H. L. Behrman, editors. Academic
14. Kuzuya, H., P. M. Blix, D. L. Horowitz, D. R. Steiner, and
A. H. Rubenstein. 1977. Determination of free and total insulin and
15. Varandani, P. T. 1967. Studies on the nature of antigenicity of
A and B chains of bovine insulin. Biochemistry. 6:100–104.
Acad. Sci. 121:404–422.
17. Faber, O. K., C. Binder, J. Markussen, L. G. Heding, V. K.
18. Steiner, D. F., S. Cho, P. E. Oyer, S. Terris, J. D. Petersen,
and A. H. Rubenstein. 1971. Isolation and characterization of proinsulin C-
20. Bell, G. L., R. L. Picket, W. J. Rutter, B. Cordell, E. Tischer,
(Lond.). 284:26–32.