3-Dehydroquinate Production by Oxidative Fermentation and Further Conversion of 3-Dehydroquinate to the Intermediates in the Shikimate Pathway

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Received April 7, 2003; Accepted July 24, 2003

3-Dehydroquinate production from quinate by oxidative fermentation with Gluconobacter strains of acetic acid bacteria was analyzed for the first time. In the bacterial membrane, quinate dehydrogenase, a typical quinoprotein containing pyrroloquinoline quinone (PQQ) as the coenzyme, functions as the primary enzyme in quinate oxidation. Quinate was oxidized to 3-dehydroquinate with the final yield of almost 100% in earlier growth phase. Resting cells, dried cells, and immobilized cells or an immobilized membrane fraction of Gluconobacter strains were found to be useful biocatalysts for quinate oxidation. 3-Dehydroquinate was further converted to 3-dehydroshikimate with a reasonable yield by growing cells and also immobilized cells. Strong enzyme activities of 3-dehydroquinate dehydratase and NADP-dependent shikimate dehydrogenase were detected in the soluble fraction of the same organism and partially fractionated from each other. Since the shikimate pathway is remote from glucose in the metabolic pathway, the entrance into the shikimate pathway from quinate to 3-dehydroquinate looks advantageous to produce metabolic intermediates in the shikimate pathway.

Key words: acetic acid bacteria; 3-dehydroquinate; 3-dehydroshikimate; quinate oxidation; shikimate pathway

Shikimate is a versatile intermediate in the shikimate pathway of aromatic compounds in animals and plants, and even in microorganisms.1) The shikimate pathway is important in pharmaceutical and industrial significance, because three aromatic amino acids, more than ten different antibiotics, and many biodegrading herbicides and pesticides are originated from the shikimate pathway. Besides shikimate, 3-dehydroquinate (DQA) and 3-dehydroshikimate (DSA) are also common intermediates in these pathways.2,3) In spite of the importance of the shikimate pathway to a variety of applications, little information has been accumulated about the shikimate pathway regarding interconversion of intermediates. One reason can be that the shikimate pathway is very remote from the initial substrate, glucose, and it is difficult to control the pathway by means of molecular biology. 3-Deoxy-7-phospho-D-arabinohexitolose, a key intermediate to shikimate, is produced by merging two different metabolic pathways, phosphoenolpyruvate from the glycolytic pathway, and erythrose-4-phosphate from the pentose phosphate pathway. Thus, many steps of enzymatic reactions are required before getting shikimate from glucose (Fig. 1).

Quinate utilization by microorganisms is known as an important index in the systematic determinative bacteriology for Pseudomonas, and many other aerobic bacteria. Quinate is used as a growth substrate by fungi as well as by bacteria and is metabolized via 3-dehydroquinate (DQA) and 3-dehydroshikimate (DSA). DQA dehydratase (synonymous to 5-dehydroquinate, EC 4.2.1.10) (DQD) was isolated from cell extract of Escherichia coli.5) The presence of quinate 5-dehydrogenase (synonymous to NAD(P)-dependent quinate dehydrogenase, EC 1.1.1.24) and shikimate 5-dehydrogenase (synonymous to 5-dehydroshikimate reductase, EC 1.1.1.25) (SKDH) were found in Aerobacter aerogenes5) and in E. coli.6) These enzymes were also indicated from

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Abbreviations: DQA, 3-dehydroquinate; DQD, 3-dehydroquinate dehydratase (EC 4.2.1.10); DSA, 3-dehydroshikimate; PQQ, pyrroloquinoline quinone; QDH, quinoprotein quinate dehydrogenase (EC 1.1.99.25); SKDH, NADP-dependent quinate dehydrogenase (EC 1.1.1.25)
Formation of 3-Dehydroquinate and Other Intermediates in Shikimate Pathway

The first report of a quinate-oxidizing enzyme in acetic acid bacteria was done by Whiting and Coggins. They pointed out quinate oxidation to DQA by NAD(P)-independent quinate dehydrogenase (QDH) (EC 1.1.99.25) and shikimate oxidation to DSA by an enzyme associated with the particulate enzyme in the cytoplasmic membrane. Van Kleef and Duine indicated the occurrence of QDH in the periplasm of Acinetobacter calcoaceticus LMD 79.41 and suggested that QDH is a quinoprotein in which pyrroloquinoline quinone (PQQ) is involved. Elsemore and Ornston did genetic analysis of protocatechuate catabolism and claimed that a consecutive action of three genes, quiA encoding QDH, quiB for DQD, and quiC for DSA dehydratase, are required in the catabolism of quinate to protocatechuate.

As reported elsewhere, DQA production from quinate is catalyzed by a membrane-bound QDH, and NAD-dependent quinate dehydrogenase in the cytoplasmic fraction makes no contribution to quinate oxidation. Since DQA is produced rapidly under various conditions by acetic acid bacteria, biocconversion of quinate to DQA by immobilized cells is examined. In this paper, microbial production of DQA by oxidative fermentation with acetic acid bacteria and further enzymatic conversion of DQA to DSA is briefly done.

**Materials and Methods**

**Chemicals.** NADP, NADPH, and yeast extract were kind gifts from Oriental Yeast Co. (Tokyo, Japan). Other chemicals used were from commercial sources of guaranteed grade unless otherwise stated. DQA and DSA were prepared as described in the text.

**Microorganisms and culture conditions.** Gluconobacter oxydans IFO 3244, G. oxydans IFO 3292, and G. melanogenus IFO 3294 were kind donations from the Institute for Fermentation, Osaka (IFO). The basal medium consisted of glycerol, 3 g of yeast extract, and 1 g of polypepton in 1 liter of tap water. Glycerol concentrations in the culture medium were varied up to 10 g per liter according to the conditions. If it was necessary to induce QDH in the bacterial cells in advance, 2 g of quinate was added to the culture medium and the pH of the medium was adjusted to 7.0. Microorganisms were grown in 100 ml of the medium in a 500-ml side-on 30°C with shaking. The bacterial growth was measured by a Klett-Summer photoelectric colorimeter with a red filter. For the cell-mass preparation, a seed culture in 100 ml of the medium in a 500-ml side-on flask was done overnight and transferred it to five liters of a fresh medium in a 10-L table top fermentor (MD500-10L,
Marubishi Bioengineering, Co., Tokyo, Japan), and cultivated for another 12 hr under vigorous aeration. If necessary, the five liters of culture was used as another seed culture for 40 liters of medium in a 50-L fermentor (MSJ U-50L, Marubishi Bioengineering, Co., Tokyo, Japan). The harvested cells were suspended in cold 5 mM potassium phosphate, pH 6.5, and centrifugation was repeated with a conventional centrifuge to remove materials coming from the culture medium. The precipitated cells were stored without freezing before use. Freshly harvested cells were usually used for every experiment where the intact cells were required.

Preparation of membrane fraction and cytoplasmic fraction. Cell suspension was made by homogenizing freshly harvested cells at a ratio of about 10 g wet cells per 10 ml of 5 mM potassium phosphate, pH 6.5. Trace amounts of RNase and DNase were added before cell disruption. The cell suspension was treated by passing it twice through a French pressure cell press (SIM AMINCO, Spectronic Instruments, Inc., Rochester, NY, USA) at 16,000 lb/in². After removal of intact cells by a conventional low speed centrifuge, the crude cell-free extract was further centrifuged at 68,000 g for 90 min to separate the membrane fraction from the cytoplasmic soluble fraction. The membrane fraction was washed by homogenizing the precipitate in a glass homogenizer with the same buffer and by repeating ultracentrifugation.

Enzymatic measurements of quinate, DQA, DSA, and shikimate. Quinate. Quinate in a culture medium or in an enzymatic reaction mixture was measured enzymatically using a purified QDH. Purified QDH was obtained as reported previously. Composition of the reaction mixture was essentially the same as described previously and either potassium ferricyanide or a combination of phenazine-methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP) were used as the electron acceptors.

3-Dehydroquininate. DQA was measured by reading decreasing intensity at 340 nm spectrophotometrically. First, DQA was converted to DSA by a purified DQD. DSA was then measured with SKDH in 0.1 M Tris-HCl, pH 8.0, by the addition of 0.2 μmol of NADP and arbitrary units of purified SKDH and NADPH in the total volume of 1 ml of reaction mixture. After already existing DSA in the samples had been eliminated by SKDH and NADPH, reaction was started by the addition of arbitrary units of DQD. After it was left standing for 20 min to ensure completion of the reaction at 30°C, the intensity of the reaction mixture was read and the absorbance from a control run was subtracted to give an apparent estimation of DQA.

3-Dehydroshikimate. DSA was measured enzymatically in 0.1 M potassium phosphate, pH 6.0, in the total volume of 1 ml of a reaction mixture in the presence of 0.2 μmol of NADPH and arbitrary units of purified SKDH. Reading the decrease in absorbance at 340 nm in the rate assay showed a good proportionality to the concentration of DSA.

Shikimate. Shikimate was measured enzymatically in 0.1 M glycine-NaOH, pH 10.0, in the total volume of 1 ml of a reaction mixture in the presence of 0.2 μmol of NADP and arbitrary units of purified SKDH. Reading the increase in absorbance at 340 nm in the rate assay showed a good proportionality to the concentration of shikimate.

Protocatechuolate. A qualitative detection of protocatechuolate was done by reacting DSA with DSA dehydratase. Protocatechuolate formation allowed a color change of the reaction mixture to orange having λmax at 460 nm. It was only observed when DSA dehydratase was added to a reaction mixture containing enzyme reaction mixtures yielding DSA.

Detection of quinate metabolites by paper chromatography. Paper chromatography and detection of intermediates in the shikimate pathway. Paper was developed with a solvent containing benzylalcohol:2-butanol:2-propanol:water = 3:1:1:1 (w/v) containing 2% formic acid. After this was dried up in a draft chamber, freshly prepared sodium metaphosphate (160 mg) in a mixture of each 12.5 ml of 1 M acetic acid and 1 M Na-acetate was sprayed over the paper. About 20 min later when the paper was still wet, the second coloring agent, 3% (v/v) aniline in alcohol, was sprayed over the paper according to the method described by Yoshida and Hasegawa. In most cases, quinate of which the Rf value of 0.23 gave a spot of pale pink, DQA (Rf = 0.28) gave yellow, shikimate (Rf = 0.43) gave red, and DSA (Rf = 0.54) gave yellow, as shown in the text. Due to the dual sprayings by different coloring agents while the paper was still wet, it was difficult to show a clear picture.

Immobilization of acetic acid bacteria. Conditions for cell immobilization of acetic acid bacteria were examined according to the method described by Saeki. The bacterial culture of mid-exponential growth (1 g wet cells/10 ml of 0.1 M Tris-acetate, pH 6.5) was mixed with 3% Na-alginate solution (w/v) in 0.1 M Tris-acetate buffer, pH 6.5. The optimum of the cell suspension and Na-alginate solution was 2.5 ml/7.5 ml. The mixture was dropped into a chilled solution of 8% CaCl₂ (w/v). Gentle stirring was continued for 5 hr to confirm the embedding of the cells in the Ca-alginate. Beads were collected, washed with the same buffer to remove excess CaCl₂, and stored in 0.1 M Tris-acetate buffer, pH 6.5, at 4°C until use. The immobilized cells were mixed with quinate in various ratios and the reaction was done by gentle stirring at room temperature. In order to
check the optimum immobilization, the concentration of Na-alginate, cell density, CaCl₂ concentration, and gel bead size were examined. As the optimum immobilization conditions, 1.2% Na-alginate, 3% CaCl₂, 1.5 mm beads size, of which initial viable cells were set to 6 × 10⁵/ml gel were prepared. During quinate oxidation, the viable cell numbers were increased by three orders to 10⁸−⁹/ml of the gel.

Polyacrylamide gel was also useful for cell immobilization according the method described by Das et al. The cell suspension (10 ml) was mixed with 24% acrylamide stock solution, 10% ammonium persulfate (135 μl), and N,N,N,N′,N′-tetramethyl-enediamine (35 μl). The mixture was poured to solidify into a shallow glass container. After the gel was solidified, the gel plate was cut into small cubes before use. Cell immobilization using κ-carrageenan gave unfavorable results due to unsuccessful gel formation. Dried cells of acetic acid bacteria were rather useful and the results in quinate oxidation was almost comparable to quinate oxidation with Ca-alginate immobilized cells, although the free cells must be removed after the reaction before analysis. In order to prepare dried cells, wet cells were spread over on a filter paper (Advantec-Toyo No. 2) and kept air-dried at 30°C until the wet cells were completely dried. The air-dried cells were further dried over P₂O₅ under reduced pressure in a desiccator.

Preparation of 3-dehydroquinate and 3-dehydroshikimate. Since DQA and DSA are not available commercially, standard solutions of both compounds were prepared and used for further experiments. Freshly harvested cells were mixed with sodium quinate, the mixture was kept at 30°C under vigorous shaking, and remaining quinate in the reaction mixture was checked periodically. Immediately after the quinate reaction disappeared in the reaction mixture, the cells were separated by a conventional centrifuge. The supernatant was lyophilized and stored until use. The dried materials were dissolved in a minimal volume of water and the insoluble materials were removed by centrifugation. The supernatant was put on a column (1.5 × 70 cm) of Ca-form of Dowex 50 and eluted with water. When fractionation was done every 5 ml, unreacted quinate appeared at the fraction number of 30 and DQA came out at the fraction number of 55. The fractions containing DQA were combined and lyophilized and stored before use in a dark desiccator under reduced pressure. DSA was prepared by the reaction of DQD purified from G. oxydans IFO 3244. An alternative method of DSA formation was done with dried cells. The dried cells (1 mg/ml) were put into 1% DQA solution (3 ml) in a test tube, shaken at 30°C (200 rpm), and a sample of the reaction mixture was picked up periodically.

Results and Discussion

Quinate oxidation by acetic acid bacteria

When several strains of Gluconobacter were cultured in the basal medium containing 0.1% glycerol and 0.2% quinate, quinate rapidly disappeared from the culture medium and stoichiometric amount of DQA was accumulated instead before the culture came to the mid exponential growth phase. This is the first stoichiometric observation of DQA formation by means of oxidative fermentation. DQA accumulation was strongly catalyzed by Gluconobacter strains like G. oxydans IFO 3244, G. oxydans IFO 3292, G. melanogenus IFO 3294, G. liquefaciens IFO 12388, and several isolated Gluconobacter strains. G. oxydans IFO 3244 was grown on the basal medium to the mid exponential growth phase (after 12 hr) of which Klett intensity was 40, 120, and 150 when glycerol was added to 0%, 0.1%, and 1%, respectively. When quinate was added to the culture medium to 0.2% (w/v), quinate contents in the culture media dropped rapidly to zero after three hr of incubation. When quinate oxidation was done with the resting cells, the cell concentration was adjusted to 0.75 by measuring turbidity at 600 nm. Judging from the results with resting cells, rapid quinate oxidation occurred with quinate grown cells, while a 90-min lag time was observed with the cells which had been grown in the absence of quinate. These results indicate that induction of quinate oxidase system occurs soon after addition of quinate.

Quinate oxidation by immobilized cells of acetic acid bacteria

Immobilized cells were also useful catalysts in quinate oxidation. When examined with Ca-alginate immobilized cells and polyacrylamide gel immobilized cells of G. oxydans IFO 3244, quinate oxidation was completed within 12 hr and 20 hr, respectively, under the conditions used as shown in Fig. 2. Almost the same results as above were obtained with other strains, G. oxydans IFO 3292 and G. melanogenus IFO 3294. The Ca-alginate immobilized cells showed a faster rate of quinate oxidation than polyacrylamide-gel immobilized cells. The difference may come from the differences of availability of the surface area between two carriers used for cell immobilization. It was obvious that the other factors such as the matrix size of the gel and hydrodynamic parameters must affect substrate diffusion rate through the gels. Taking all these factors into consideration, the optimized immobilization of acetic acid bacteria must be obtained to give the highest quinate oxidation to DQA.
Fig. 2. Quinate Oxidation by Ca-Alginate Immobilized Cells and Polyacrylamide Gel Immobilized Cells.

Freshly harvested cells of *G. oxydans* IFO 3244 were used for immobilization. Squares and circles mean Ca-alginate immobilized cells and polyacrylamide gel immobilized cells, respectively. Immobilized cells were mixed with 10 mg/ml of quinate and gently shaken (90 rpm) for the period indicated. Quinate in the reaction mixture was measured periodically under the standard assay conditions.

Fig. 3. 3-Dehydroquinate Oxidation by Immobilized Cells of Acetic Acid Bacteria.

Ca-Alginate immobilized cells of *Gluconobacter* strains were incubated with 30 mg DQA in 3 ml with shaking at 30°C for the period as indicated. The numbers shown in the figure correspond to the strain: 3244, *G. oxydans* IFO 3244; 3292, *G. oxydans* IFO 3292; 3294, *G. melanogenus* IFO 3294. Samples of the reaction mixture were taken periodically for the measurement of DSA.

3-Dehydroshikimate production from quinate and 3-dehydroquinate

DSA production by Ca-alginate immobilized cells of *G. oxydans* IFO 3244, *G. oxydans* IFO 3292, and *G. melanogenus* IFO 3294 proceeded with 11, 10, and 8.4% of final yield, respectively, when DQA was used as the initial substrate (Fig. 3). When DSA production was done with quinate, the total yield of DSA was lower than those examined with DQA as the substrate (data not shown). The unexpected low yield of DSA might come from the divergent metabolic pathways of the product, some of the DSA produced may be converted without accumulating in the reaction mixture. This was suggested by the following experiments. When dried cells and freshly harvested wet cells were tested for DSA production, DSA was clearly detected in the reaction mixture including dried cells, while a scarce amount of DSA was detected in the reaction mixture examined with freshly harvested cells (Fig. 4). Effectiveness and usefulness of dried cells for the production of metabolic intermediates by means of bioconversion has been described in other cases.20) Permeability of substrate and product through the cytoplasmic membranes becomes important in the experiment where whole cells were used as biocatalyst. Anyway, it was something far from a stoichiometric account when compared to DQA production by oxidative fermentation, and DSA may be controlled by the cytoplasmic metabolism. The DSA produced may not stay stable and may be further converted to shikimate and protocatechuate. In fact, in the reaction mixture in which dried cells or immobilized cells were reacted with DQA, shikimate and protocatechuate were always detected more or less in addition to DSA.

Chromatographic detection of oxidation products

To avoid heavy tailing in paper development, resting cells were prepared from freshly harvested cells and directly used for quinate oxidation at the final cell density of 0.75 at 600 nm. As shown in Fig. 5, quinate initially loaded at 10 mg/ml was converted to DQA within 12 hr of reaction. After the same situation was maintained for another 12 hr until quinate was completely converted to DQA, DQA was gradually converted to DSA in the next 24 hr. After 72 hr
Fig. 5. Chromatographic Detection of Oxidation Products of Quinate.

Dried cells of *G. oxydans* IFO 3244 (1 mg/ml) were mixed with 10 mg/ml of quinate and shaken at 30°C for the period indicated. Samples of the reaction mixture were taken periodically and spotted on a paper. Other chromatographic conditions are given in Materials and Methods.

of incubation, almost equal amounts of DQA and DSA were detected in the reaction mixture. Since acetic acid bacteria catalyze a one-step incomplete oxidation reaction in the oxidative fermentation, it is advantageous to collect the oxidation product by controlling the reaction conditions. Thus, QDA production by acetic acid bacteria was shown here for the first time by criteria using growing cells, immobilized cells, and dried cells. If the cells were removed from the reaction mixture after quinate depletion, DQA can be collected with the highest yield. Other oxidative bacteria like *Pseudomonas* strains or *Acinetobacter* strains also contain QDH oxidizing quinate to DQA. However, since these aerobic bacteria contain strong assimilating enzymes like DSA dehydratase which leads DSA catabolism to succinate and acetyl-CoA via protocatechuate; such bacteria do not accumulate DQA and also DSA as high as acetic acid bacteria.

**Mutual separation of SKDH, DQD, and SDA dehydratase**

The cytoplasmic fraction of *G. oxydans* IFO 3244 was prepared from 90 g of wet cells and put on a DEAE-cellulose column (2 × 25 cm), which had been equilibrated with 2 mM potassium phosphate, pH 6.5, containing 5 mM β-mercaptoethanol (Buffer A). After the column was washed with Buffer A, SKDH was eluted from the column with Buffer A containing 0.15 M KCl and DQD came out from the column with Buffer A containing 0.25 M KCl. SKDH was further purified by a DEAE-Sephadex A-50 column (1.2 × 20 cm), which had been equilibrated with buffer A. Elution of SKDH was done by a linear gradient of KCl concentration between 500 ml of Buffer A containing 0.03 M KCl and 500 ml of Buffer A containing 0.11 M KCl. Judging from the elution pattern (not shown), SKDH came out at about 0.075 M KCl concentration. The partially purified SKDH at this stage contained no enzyme activities of DQD and DSA dehydratase, though several minor protein bands were still appeared in polyacrylamide gel electrophoresis (data not shown). In further purification of DQD, the impurity was then removed by a hydroxypatite column (5 × 10 cm) equilibrated with Buffer A, to which DQD was adsorbed while the major impurity was not adsorbed under these conditions. DQD was eluted from the column with Buffer A of which potassium phosphate was increased to 50 mM. The dialyzed DQD was put on a small DEAE-Sephadex A-50 column (1 × 10 cm), which had been equilibrated with the same Buffer A used for enzyme dialysis. After the column was washed with Buffer A containing 0.175 M KCl, DQD was finally eluted from the column with Buffer A containing 0.2 M KCl. During DQD purification, the enzyme activity of DSA dehydratase was separated from DQD at the final step of chromatography. DQD was eluted from the column under the conditions above, whereas DSA dehydratase was eluted from the column only when KCl concentration in Buffer A was increased to 0.225 M KCl. A manuscript on the overall purification of SKDH and DQD is now being prepared.

**Preliminary production of shikimate by a single-cellular system**

As mentioned above, characterization of the enzymes relating to enzymatic conversion of quinate to shikimate was done in this study. In the case of dried cell experiments, a positive SKDH reaction was detected by increased absorbance at 340 nm with a sample of the reaction mixture when shikimate production was measured with SKDH in the presence of NADP. This strongly indicates that shikimate production by the single-cellular system of acetic acid bacteria would be possible, although the reaction conditions or water contents in the dried cells must be optimized until the data become reproducible and convincing. To confirm shikimate formation by the single-cellular system of acetic acid bacteria, quinate was oxidized first to DQA, which must be coupled with DQD and then with SKDH in the presence of NADPH at pH 7.0. Dried cells (3 mg) were poured into a 3-ml reaction mixture containing 30 mg DQA and shaken on a rotary shaker (200 rpm) at 30°C. Samples were taken periodically for the SKDH reaction. Measurement of shikimate produced was done with SKDH and NADP in glycine-NAOH, pH 10.0, and shikimate production was almost proportional to reaction time and DQA concentration. A similar system was reconstructed *in vitro* in the presence of
DQD and SKDH, where DQA was given as the starting substrate. DQA was the preferential substrate to quinate in any experiments. In addition to NADP, additions of NADP-dependent glucose dehydrogenase and excess glucose (100 μmol) confirmed shikimate formation as schematically shown in Fig. 6. Any enzyme yielding NADPH, like d-glucose-6-phosphate dehydrogenase or 6-phospho-d-glucuronate dehydrogenase from Gluconobacter strains can be coupled as NADPH generating enzyme. D-Glucose dehydrogenase must be suitable for routine purposes because of the expenses of glucose. As written above, DSA dehydratase was separated from DQD. One strategy to pull this system to shikimate production is genetic deletion or inactivation of DQA from DQD. One strategy to pull this system to shikimate production is genetic deletion or inactivation of DQA from DQD. Any enzyme yielding NADPH, like D-α-mannoside reductase, is highly acknowledged. Grant support to O.A. from the Uehara Life Science Foundation for 2002 was also grateful.

Acknowledgments

A part of this work was done under collaboration in the Core University Program between Yamaguchi University and Kasetsart University supported by the Scientific Cooperation Program from the Japan Society for the Promotion of Science (JSPS) and the National Research Council of Thailand (NRCT). Technical contributions by Mr. Tomonori Funahashi are highly acknowledged. Grant support to O.A. from the Uehara Life Science Foundation for 2002 was also grateful.

References

15) Tanasupawat, S., Adachi, O., Yoshihara, N., Toyama, H., and Matsushita, K., Purification and characterization of quinoprotein quinate de-
hydrogenase, in the proceedings of the 2nd Joint Seminar of JSPS-NRCT on Thermotolerant Microbial Resources, Nov. 21–25, p. 36 (2000).


