as demonstrated in Figure 5. The rapid degradation of linear DNA to smaller fragment suggests that other double-strand breaks might also be formed in a similar manner, with random cleavage of one strand of duplex DNA followed by a separate cleavage event in close proximity on the opposite strand to yield fragments. It is not necessary to invoke a model using molecular dimers of bleomycin to account for rapid accumulation of double-strand breaks; a preference for nicked sites would be sufficient.

References


Berkner, K. L., & Folk, W. R. (1977) *J. Biol. Chem. 252*, 3176.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608.

Goff, S., & Rambach, A. (1978) *Gene 3*, 347.

Haidle, C. W. (1971) *Mol. Pharmacol. 7*, 645.

Haidle, C. W., Weiss, K. K., & Kuo, M. T. (1972) *Mol. Pharmacol. 8*, 531.

Ishida, R., & Takahashi, T. (1976) *Biochem. Biophys. Res. Commun. 60*, 1432.

Kuhnlein, U., Penhoet, E. E., & Linn, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1169.

Kuo, M. T., & Haidle, C. W. (1973) *Biochim. Biophys. Acta 335*, 109.

Lloyd, R. S., Haidle, C. W., & Robberson, D. L. (1978a) *Biochemistry 17*, 1890.

Lloyd, R. S., Haidle, C. W., Robberson, D. L., & Dodson, M. L., Jr. (1978b) *Curr. Microbiol. 1*, 45.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560.

Muller, W. E. G., & Zahn, R. K. (1976) in *Gann Monograph on Cancer Research No. 19. Fundamental and Clinical Studies of Bleomycin* (Carter, S. K., Ichikawa, T., Mathe, G., & Umegawa, H., Eds.) pp 51, University Park Press, Baltimore, MD.

Paoletti, C., LePecq, J. B., & Lehman, I. R. (1971) *J. Mol. Biol. 55*, 75.

Poon, R., Beerman, T. A., & Goldberg, I. H. (1977) *Biochemistry 16*, 486.

Povrik, L. F., Wubker, W., Kohnlein, W., & Hutchinson, F. (1977) *Nucleic Acids Res. 4*, 3573.

Ross, S. L., & Moses, R. E. (1978) *Biochemistry 17*, 581.

Sanger, F., Air, G. M., Barrell, B. G., Brown, N. L., Coulson, A. R., Fiddes, J. C., Hutchison, C. A., Slocombe, P. M., & Smith, M. (1977) *Nature (London) 265*, 687.

Schekman, R. W., Iwaya, M., Bromstrup, K., & Denhardt, D. I. (1971) *J. Mol. Biol. 57*, 117.

Shapiro, H. S., & Chargaff, E. (1964) *Biochim. Biophys. Acta 91*, 262.

Smith, D. E., Blattner, F. R., & Davies, J. (1976) *Nucleic Acids Res. 3*, 343.

Suzuki, H., Nagi, K., Yamaki, H., Tanaka, N., & Umezawa, H. (1969) *J. Antibiot. 22*, 446.

Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983.

Umezawa, H. (1975) in *Antibiotics. Mechanism of Action of Antimicrobial and Antitumor Agents* (Corcoran, J. W., & Han, F. E., Eds.) Vol. III, pp 21, Springer-Verlag, New York.

Umezawa, H., Asakura, H., Oda, K., Hori, S., & Hort, M. (1973) *J. Antibiot. 26*, 521.


# Biosynthesis of *o*-Succinylbenzoic Acid in a *men*⁻ *Escherichia coli* Mutant Requires Decarboxylation of L-Glutamate at the C-1 Position†

R. Meganathan and Ronald Bentley*

ABSTRACT: A *men*⁻ mutant of *Escherichia coli*, AN 209, which accumulates *o*-succinylbenzoic acid, has been used for a direct study of the biosynthesis of this benzenoid compound. Samples of labeled glutamic acids were added to growth media, and the *o*-succinylbenzoic acid was isolated and converted to a dimethyl derivative. This dimethyl derivative was purified on thin-layer chromatograms and by gas chromatography. When the glutamic acid used as precursor contained $^{14}C$ at position 5, or was uniformly labeled, the dimethyl *o*-succinylbenzoate contained radioactivity (as shown by radiogas chromatography). However, from [1-$^{14}C$]glutamate, the dimethyl *o*-succinylbenzoate was without radioactivity. Hence, in the biosynthesis of *o*-succinylbenzoate, carbon atom 1 of glutamate is lost, and carbon atoms 2–5 are retained. It was also shown that this mutant lacked the enzyme dihydroxynaphthoic acid synthase. It should, therefore, continue to be classified as a *menB* mutant, rather than as a member of the newly created *menE* group (lacking *o*-succinylbenzoate-CoA synthetase).

That menaquinones are shikimate-derived materials has been known since the early experiments of Cox & Gibson (1964, 1966). Seven of the ten carbon atoms of the naphthalenoid nucleus are derived from the intact shikimate skeleton (Campbell et al., 1967, 1971; Guêrin et al., 1970); the remaining three atoms originate in either $\alpha$-ketoglutarate or glutamate with both of the carboxyl carbon atoms of these precursors being lost (Robins et al, 1970; Campbell et al., 1971; Robins & Bentley, 1972). To account for the observed role of the $C_5$ compounds, Campbell (1969) proposed that a thiamin pyrophosphate complex of succinic semialdehyde (formed from $\alpha$-ketoglutarate) condensed with shikimate in a reaction analogous to the Michael addition. Subsequent evidence implicated chorismate rather than shikimate as the material reacting with the $C_5$ precursor; it was also suggested that *o*-succinylbenzoic acid (OSB)[1] was a product of the conden-

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sation (Dansette & Azerad, 1970). Experiments with labeled OSB showed that it was incorporated into menaquinones with loss of the side chain carboxyl group (Dansette & Azerad, 1970; Campbell et al., 1971; Robins et al., 1973). OSB is further converted to the naphthalenoid compound 1,4-dihydroxy-2-naphthoic acid (DHNA) as postulated by Robins et al. (1970); this process is CoA and ATP dependent and requires the action of two enzymes (Meganathan & Bentley, 1979; Meganathan et al., 1980). The subsequent reactions leading from DHNA to menaquinones are relatively straightforward, and enzymes carrying out methylation and prenylation reactions have been obtained (Samuel & Azerad, 1972; Shineberg & Young, 1976).

Depsite this strong evidence for the sequence shikimate → chorismate → OSB → OSB-CoA → DHNA → MK, there has been no direct study of the biosynthesis of OSB itself. It is not accumulated to any significant extent in the culture media of wild-type strains of bacteria, and there is only minimal evidence for the presence of small amounts of this metabolite in plants (Grotzinger & Campbell, 1974). We have used a menaquinone-deficient mutant of *Escherichia coli*, which accumulates OSB, to examine directly the incorporation of carbon atoms from glutamate into this interesting benzenoid derivative. In addition, we have pinpointed the enzymatic lesion in this mutant.

## Materials and Methods

*Growth Conditions for Tracer Experiments.* A menaquinone-deficient mutant, AN 209, of *Escherichia coli* isolated by Young (1975) was used in this work. It was originally described as a *menB* mutant (*menB404 metB*$^-$) being blocked in the conversion of OSB to DHNA; the exact nature of this block will be clarified in detail under Results. The minimal growth medium and the concentrations of supplements used in our experiments are the same as those described by Stroobant et al. (1972). Portions (50 mL) of this minimal medium were inoculated and grown overnight at 37 °C with shaking (Gyrotary shaker, 200 rpm). In turn, this culture was used to inoculate 950-mL portions of the same medium, to which were added the variously labeled samples of glutamic acid. These large-scale cultures were grown until the absorbance at 700 nm reached 1.2–1.4.

*Isolation and Derivatization of the Benzenoid Product.* Following growth, the culture was centrifuged at 4500 rpm and 4 °C for 10 min in the Sorvall RC3 centrifuge using the HG-4 rotor. The supernatant was concentrated to 80 mL in a rotary evaporator at 50 °C and acidified to pH 2 with concentrated HCl. After being cooled in an ice bath, the mixture was centrifuged at 15 000 rpm (Sorvall RC5-B centrifuge, SA 600 rotor) for 15 min at 4 °C, and the precipitate was discarded. The volume of the supernatant solution was made up to 100 mL with water, and extraction with three 100-mL portions of ethyl acetate was carried out. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, then filtered, and evaporated to dryness in a rotary evaporator. The residue was treated with 12% NaOH (10 mL) for 10 min at room temperature to convert any OSB spirodilactone to OSB. After the pH was adjusted to 2 with concentrated HCl, the volume was brought to 20 mL, and the mixture was extracted 3 times with an equal volume of ethyl acetate. The extracts were dried (sodium sulfate), filtered, and evaporated to dryness under vacuum. The residue was converted to a methyl derivative by solution in 1 mL of methanol and treatment with an excess of diazomethane in ether at room temperature for 30 min. After evaporation in a rotary evaporator, the residue was dissolved in 500 μL of ethyl acetate for thin-layer chromatography on Anasil OF plates; the solvent was benzene:ethyl acetate:acetic acid, 150:75:2. Authentic dimethyl OSB was used as a control, and the materials were located by visualization in UV light. Material with the same $R_f$ value as authentic dimethyl OSB was scraped from the plate and suspended in 4 mL of 0.2 N HCl. This mixture was extracted 3 times with an equal volume of ethyl acetate. The combined extracts were evaporated to dryness under vacuum; the residue was dissolved in 500 μL of ethyl acetate, and this material was again subjected to thin-layer chromatography in the same solvent system. These chromatograms showed a single spot (when visualized under UV light) which had the same $R_f$ value as that of authentic dimethyl OSB. The plates were dried and were then scanned for radioactivity in the Packard scanner, Model 7201.

For the final examination, the spot of dimethyl OSB was again scraped off the thin-layer plate, and after elution with 0.2 N HCl and extraction with ethyl acetate, the material was examined by combined gas chromatography–proportional counting (Campbell, 1979).

*Preparation and Assay of Enzyme Extracts.* For these experiments, *E. coli*, AN 209, was inoculated into 50-mL portions of trypticase soy broth medium (Baltimore Biological Laboratories, Cockeysville, MD) and was allowed to grow overnight at 37 °C with shaking. These cultures were used to inoculate 950-mL portions of the same medium in 2.8-L Fernbach flasks. After growth at 37 °C for 7 h (Gyrotary shaker), the cells were harvested by centrifugation (Sorvall RC-3 centrifuge, HG-4 rotor, 4500 rpm, 4 °C, 15 min). The cells were resuspended in 300 mL of 0.02 M potassium phosphate buffer, pH 6.9, and were then centrifuged again as just described. The cell paste was stored in frozen condition at −20 °C.

The frozen cell paste (5 g) was suspended in 7.5 mL of 0.02 M potassium phosphate buffer, pH 6.9, which also contained 5 mM mercaptoethanol. This suspension was passed through a French press at 12 000 psi. The resulting mixture was centrifuged (Sorvall RC-5B centrifuge, SA-600 rotor) at 15 000 rpm, 4 °C, for 30 min. The supernatant from this centrifugation was used without further treatment. Complementation assays with OSB-CoA synthetase and DHNA synthase were carried out as described previously at pH 8.0 (Meganathan et al., 1981). Protein determinations were carried out by the method of Bradford (1976) using reagents and protein standards supplied by Bio-Rad Laboratories, Richmond, CA.

*Chemicals.* L-[U-$^{14}$C]Glutamic acid was obtained from ICN Chemical and Radioisotope Division (Irvine, CA), L-[1-$^{14}$C]glutamic acid was from New England Nuclear (Boston, MA), and DL-[5-$^{14}$C]glutamic acid was from Research Products International (Elk Grove Village, IL). [2,3-$^{14}C_2$]OSB and nonlabeled OSB were prepared as previously described (Meganathan et al., 1981). Anasil OF thin-layer chromatographic plates were a product of Analabs, (North Haven, CT), and silica gel GF plates were from Analtech (Newark, DE). All other chemicals were of the highest quality available, and all solvents were redistilled prior to use. The authentic sample of the dimethyl OSB was prepared by dissolving OSB (5 mg)

[1] Abbreviations used: OSB, *o*-succinylbenzoic acid [4-(2-carboxyphenyl)-4-oxobutyric acid]; DHNA, 1,4-dihydroxy-2-naphthoic acid; MK, menaquinone; DMK, demethylmenaquinone; CoA, coenzyme A. Since this paper is concerned only in a peripheral way with the menaquinones and demethylmenaquinones, the abbreviations MK and DMK are used in a general sense, without defining the number of isoprene units in the side chain.

Table I: Identification of the Enzymatic Defect in *E. coli* AN 209[a]

| incubation mixture | DHNA production (nmol formed/ 30 min) |
|---|---|
| AN 209 extract | <0.05 |
| AN 209 extract + OSB-CoA synthetase | <0.05 |
| OSB-CoA synthetase | <0.05 |
| AN 209 extract + DHNA synthase | 9.3 |
| DHNA synthase | 0.4 |

[a] The total incubation volume was 3.0 mL and was made up with 0.1 M potassium phosphate buffer, pH 8, containing 5 mM mercaptoethanol. The volume of AN 209 extract used was 0.2 mL; this solution contained 15.8 mg of protein. In the complementation experiments, the tubes contained either 7.4 units of OSB-CoA synthetase (in a volume of 0.05 mL) or 17.2 units of DHNA synthase (in a volume of 0.2 mL). Each tube also contained the following (amounts in micromoles): OSB, 0.25; CoA, 0.5; ATP, 4.8; $MgCl_2$, 20. After a 30-min incubation at 30 °C, the DHNA produced was determined by the spectrophotofluorometric assay (Meganathan & Bentley, 1979).

in methanol (2 mL) and adding an excess of ethereal diazomethane. After 30 min at room temperature, solvents and excess diazomethane were removed by vacuum evaporation. The diazomethane was prepared from Diazald (Aldrich Chemical Co., Milwaukee, WI).

## Results

The *E. coli* mutant, AN 209, used in this work was isolated by Young (1975). It was blocked in the conversion of OSB to DHNA and at that time was described as a *menB* mutant (*menB404 metB*⁻). As a result of the enzymatic defect, OSB was excreted into the culture medium. It has now been shown that the conversion, OSB → DHNA, requires two enzymes designatged as OSB-CoA synthetase and DHNA synthase (Meganathan & Bentley, 1979; Meganathan et al., 1980). The formation of DHNA is more completely represented as follows: OSB → OSB-CoA → DHNA. It was, therefore, necessary to characterize the AN 209 mutant more completely. Cell-free extracts were prepared (see Materials and Methods) and were complemented with either OSB-CoA synthetase or DHNA synthase prepared from *Mycobacterium phlei*; the mixtures were then incubated with OSB, CoA, and ATP, and the yield of DHNA was determined. As shown in Table I, extracts of the *E. coli* mutant produced DHNA only when complemented with DHNA synthase; hence, the enzymatic defect was pinpointed as a specific lack of DHNA synthase. In conformity with the revised nomenclature for *men* mutants of *Bacillus subtilis* (Meganathan et al., 1981), the designation *menB* will be retained for this and other mutants lacking DHNA synthase. Mutants lacking OSB-CoA synthetase are now termed *menE*.

The CoA derivative of OSB appears to be a rather unstable material (McGovern & Bentley, 1978; Hutson & Threlfall, 1978; Meganathan & Bentley, 1979; Meganathan et al., 1980) and readily undergoes a cyclization to a spirodilactone form of OSB (with elimination of CoA). This cyclization is particularly marked in extracts of organisms that are depleted of the DHNA synthase. It was, therefore, to be expected that cell-free extracts of the *menB* mutant would accumulate the spirodilactone of OSB. When extracts of AN 209 were prepared by use of the French press and were then incubated with [2,3-$^{14}C_2$]OSB, the presence of the dilactone was confirmed by scanning of thin-layer chromatograms (Figure 1).

To study the utilization of the carbon atoms of glutamate for the biosynthesis of OSB, we grew AN 209 cultures in the

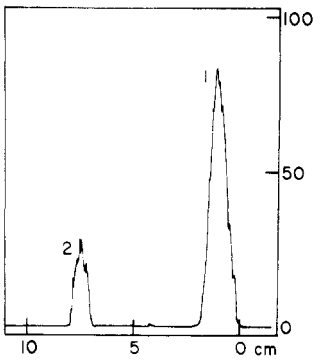


FIGURE 1: Identification of spirodilactone form of OSB after incubation of [2,3-$^{14}C_2$]OSB with enzyme extracts from *E. coli* AN 209. The cell-free extract prepared from *E. coli* AN 209 (0.2 mL, 15.8 mg of protein) was incubated under the usual assay conditions (Meganathan et al., 1981) with potassium phosphate buffer at pH 8.0; the "cold" OSB was replaced by [2,3-$^{14}C_2$]OSB (100 000 dpm). After being incubated for 30 min at 30 °C, the reaction was terminated by addition of 3 mL of acetone:concentrated HCl, 100:1. This mixture was extracted with benzene (3 mL), and the upper layer was separated and evaporated to dryness in a rotary evaporator. The residue was dissolved in 500 μL of ethyl acetate, and this solution was streaked on a silica gel GF plate. After development in chloroform:ethyl acetate:formic acid, 135:20:1.5, for a distance of 17.5 cm, the plate was scanned for radioactivity in the Packard radiochromatogram scanner. The ordinate of the figure shows the percentage of full-scale deflection of the instrument. Instrument settings were the following: range, 1 × $10^3$; speed, 1 cm/min; time constant, 10 s; slit width, 2 mm. The O-cm mark on the abscissa indicates the "origin" of the thin-layer chromatogram. The peaks of radioactivity correspond precisely with the positions of standard materials; they are identified as (1) OSB and (2) spirodilactone of OSB.

presence of either L-[U-$^{14}$C]-, L-[1-$^{14}$C]-, or DL-[5-$^{14}$C]-glutamate; the amounts of radioactivity added were 250 μCi with the L enantiomeric forms and 500 μCi when the racemic material was used. The increased amount of DL-[5-$^{14}$C]-glutamate was used in view of the uncertainty regarding the utilization of D-glutamate in this *E. coli* mutant; glutamate racemase does not appear to be a widely distributed enzyme (Adams, 1972). The radioactive glutamates were added at the time of inoculation into the large flasks. The cultures were harvested 8–10 h later. The OSB in the culture supernatant was extracted, derivatized, and examined by thin-layer chromatography; the chromatograms revealed a single spot when examined under UV light. When the chromatograms were scanned to detect the presence of radioactivity, a radioactive peak, corresponding precisely with the position of dimethyl OSB, was obtained in the experiments using [U-$^{14}$C]- and [5-$^{14}$C]glutamic acid (Figure 2). When [1-$^{14}$C]glutamic acid was used as the precursor under the same conditions, the dimethyl OSB contained a much smaller level of activity (Figure 2).

A more definitive result was obtained when the products were further examined by radiogas chromatography (Campbell, 1979). The product from the experiment using [U-$^{14}$C]glutamic acid as the precursor showed two pronounced peaks of radioactivity under these conditions (Figure 3). Of these two peaks, one corresponds to the mass peak of authentic dimethyl OSB. The other radioactive peak is a contaminant which has similar thin-layer chromatographic properties to those of dimethyl OSB.

When the compound isolated from the culture treated with [5-$^{14}$C]glutamate was examined by radiogas chromatography, the pattern observed was identical with that obtained with [U-$^{14}$C]glutamate (Figure 3). However, in the experiment with [1-$^{14}$C]glutamate, there was essentially no radioactive peak corresponding to the mass peak of dimethyl OSB. These

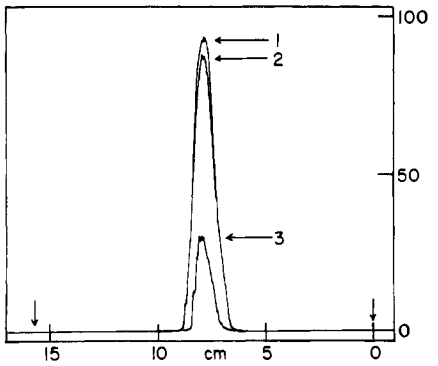


FIGURE 2: Formation of OSB from glutamate by cultures of *E. coli* AN 209. The OSB was isolated from the culture supernatants, converted to the dimethyl derivative, and purified by thin-layer chromatography as described under Materials and Methods. The final thin-layer chromatograms were scanned for the presence of radioactivity. Tracings of the scans from the three separate experiments have been superimposed in this figure for convenience. The ordinate shows the percentage of full-scale deflection of the radioactivity scanner. The instrument settings were the following: range, $3 \times 10^3$; speed, 1 cm/min; time constant, 10 s; slit width, 2 mm. The same settings were used in each of the three scans. The arrows indicate the origin of the thin-layer chromatogram (at 0 cm) and the position of the solvent front. The three samples are identified as follows: (1) dimethyl OSB obtained from the DL-[5-$^{14}$C]glutamate experiment; (2) dimethyl OSB obtained from the L-[U-$^{14}$C]glutamate experiment; (3) dimethyl OSB obtained from the L-[1-$^{14}$C]glutamate experiment. As noted under Materials and Methods, the isolation procedure included a treatment with NaOH to convert any spirodilactone to OSB itself. No spirodilactone peaks were observed on these chromatograms. The fact that the peaks from experiment 1 and 2 are of approximately the same height suggests that utilization of the D enantiomer had not occurred when DL-[5-$^{14}$C]glutamate was used (500 $\mu$Ci used in this case, but 250 $\mu$Ci of L-[U-$^{14}$C]glutamate).

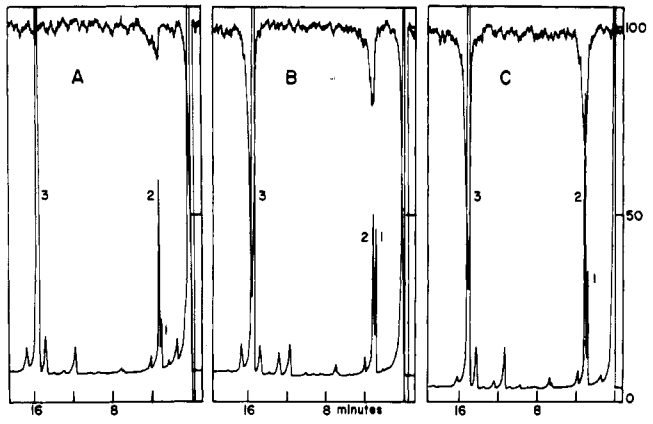


FIGURE 3: Radiogas chromatography of OSB samples biosynthesized from labeled glutamates by *E. coli*, AN 209. The dimethyl OSB samples were recovered from thin-layer chromatograms as described under Materials and Methods. They were then subjected to radiogas chromatography. The column used (6 ft × 4 mm) contained 3% SP 2250 on Supelcoport 80/100. The temperature was programmed from an initial 1-min hold at 150 °C to a final hold at 270 °C, using an increase of 5 °C/min. The lower traces show output from the flame ionization detector (sensitivity setting of $16 \times 10^{-11}$ A) and the upper traces the output from the proportional counter. For both traces, the ordinate scale is from 0 to 100% of full-scale deflection. For the proportional counter, 100% full-scale deflection represents 1000 cpm. The peaks numbered 1 and 2 represent unidentified contaminants; peak 3 corresponds to the position given by authentic dimethyl OSB. (A) Sample from culture treated with L-[1-$^{14}$C]glutamate; (B) sample from culture treated with DL-[5-$^{14}$C]glutamate; (C) sample from culture treated with L-[U-$^{14}$C]glutamate.

observations indicate that in the synthesis of OSB, the first carbon atom (C-1) of glutamate is lost. As a final control, the identity of the compound isolated from the [1-$^{14}$C]-glutamate experiment was confirmed by mass spectrometry. The mass spectrum was identical with that obtained with an

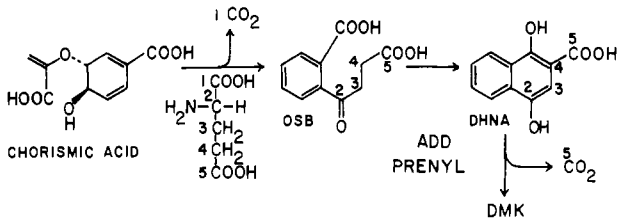


FIGURE 4: Distribution of glutamate carbon atoms in OSB and DHNA. In this figure, the numbers used with the OSB and DHNA structures refer only to the numbers of carbon atoms in glutamate. The loss of the three-carbon side chain of chorismate, which is also necessary, has not been indicated in this figure to emphasize the events relating to glutamate. The prenylation of DHNA leads to demethylmenaquinone (DMK) and loss of the carbon-5 atom of glutamate.

authentic sample of dimethyl OSB; no parent molecular ion, three largest ions $m/e$ 163 > 77 > 159 [corresponding to $C_6H_5(CO)COOCH_3$, $C_6H_5$, and $C_{10}H_7O_2$].

## Discussion

The experiments reported here show that radioactivity from either [U-$^{14}$C]- or [5-$^{14}$C]glutamate is incorporated into the OSB accumulated by the *menB E. coli* mutant AN 209. However, when [1-$^{14}$C]glutamate was used as a precursor under the same experimental conditions, the OSB was without radioactivity. Hence, in the biosynthesis of OSB, only carbon atoms 2–5 from glutamate are utilized, as indicated in Figure 4; carbon atom 1 is lost by a decarboxylation. These results are compatible with the earlier observation that the carboxyl group of the succinyl side chain of OSB is lost during the formation of menaquinones, and the three central carbon atoms of glutamate are retained (Campbell et al., 1971). Thus, when glutamate is utilized in menaquinone biosynthesis, two decarboxylations are required. The latter of these decarboxylative steps is believed to occur at the time of the prenylation reaction, or immediately thereafter (Shineberg & Young, 1976). The initial step required for OSB biosynthesis has been postulated to occur by way of the thiamin pyrophosphate adduct of succinic semialdehyde (Campbell, 1969). If true, this reaction would require the deamination of glutamate to $\alpha$-ketoglutarate. Other possible reaction schemes have been proposed (Bentley & Campbell, 1974), and at the moment it is not possible to state whether $\alpha$-ketoglutarate or glutamate is the immediate precursor to OSB.

It will be recalled that with the mutant discussed here, Young (1975) had identified free OSB in the culture fluid. Presumably, this free acid was derived by hydrolysis of the spirodilactone form. In detailed studies, Hutson & Threlfall (1978) have, in fact, shown that this hydrolytic conversion is very rapid, particularly at pH values greater than 7.

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## References

Adams, E. (1972) *Enzymes, 3rd Ed. 6*, 479–507.

Bentley, R., & Campbell, I. M. (1974) in *The Chemistry of the Quinonoid Compounds* (Patai, S., Ed.) pp 683–736, Wiley, New York.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Campbell, I. M. (1969) *Tetrahedron Lett.*, 4777–4780.

Campbell, I. M. (1979) *Anal. Chem. 51*, 1012A–1019A.
Campbell, I. M., Coscia, C. J., Kelsey, M., & Bentley, R. (1967) *Biochem. Biophys. Res. Commun. 28*, 25–29.
Campbell, I. M., Robins, D. J., Kelsey, M., & Bentley, R. (1971) *Biochemistry 10*, 3069–3078.
Cox, G. B., & Gibson, F. (1964) *Biochim. Biophys. Acta 93*, 204–206.
Cox, G. B., & Gibson, F. (1966) *Biochem. J. 100*, 1–6.
Dansette, P., & Azerad, R. (1970) *Biochem. Biophys. Res. Commun. 40*, 1090–1095.
Grotzinger, E., & Campbell, I. M. (1974) *Phytochemistry 13*, 923–926.
Guérin, M., Leduc, M. M., & Azerad, R. G. (1970) *Eur. J. Biochem. 15*, 421–427.
Hutson, K. G., & Threlfall, D. R. (1978) *Biochim. Biophys. Acta 530*, 1–8.
McGovern, E. P., & Bentley, R. (1978) *Arch. Biochem. Biophys. 188*, 56–63.
Meganathan, R., & Bentley, R. (1979) *J. Bacteriol. 140*, 92–98.
Meganathan, R., Folger, T., & Bentley, R. (1980) *Biochemistry 19*, 785–789.
Meganathan, R., Bentley, R., & Taber, H. (1981) *J. Bacteriol. 145*, 328–332.
Robins, D. J., & Bentley, R. (1972) *J. Chem. Soc., Chem. Commun.*, 232–233.
Robins, D. J., Campbell, I. M., & Bentley, R. (1970) *Biochem. Biophys. Res. Commun. 39*, 1081–1086.
Robins, D. J., Yee, R. B., & Bentley, R. (1973) *J. Bacteriol. 116*, 965–971.
Samuel, O., & Azerad, R. (1972) *Biochimie 54*, 305–317.
Shineberg, B., & Young, I. G. (1976) *Biochemistry 15*, 2754–2758.
Stroobant, P., Young, I. G., & Gibson, F. (1972) *J. Bacteriol. 109*, 134–139.
Young, I. G. (1975) *Biochemistry 15*, 399–406.

# Interaction of Hemagglutinating Virus of Japan with Erythrocytes As Studied by Release of a Spin Probe from Virus†

Toyozo Maeda,‡ Kazumichi Kuroda, Sakuji Toyama, and Shun-ichi Ohnishi*

ABSTRACT: The spin probe tempocholine was incorporated into hemagglutinating virus of Japan (HVJ) and its release on interaction with erythrocytes or ghosts studied. Two methods were used for the assay of release. One is to incorporate the probe into virus at a high concentration (~30 mM) and use the increase in the ESR peak height due to drastic weakening of the spin–spin exchange interaction on dilution. The other is to load the virus with a low concentration of tempocholine. The tempocholine remaining inside the virus was determined by reducing outside tempocholine with ascorbate. The results showed that tempocholine was rapidly released on interaction with both erythrocytes and ghosts. Requirement of the viral F glycoprotein for the release was demonstrated by using trypsinized HVJ, which has cleaved F protein, and also by using HVJ grown in cultured cells, which has the precursor form of F protein. The release was independent of the viral dose and occurred to the same extent with intact erythrocytes as with ghosts. The release from trypsinized HVJ was not enhanced by coadsorbed intact HVJ. These characteristics for the release were markedly different from those for the phospholipid transfer from HVJ envelope to erythrocyte membrane. Combining the results for the spin probe release and the phospholipid transfer has suggested that the envelope fusion is not autocatalytically accelerated, is independent of the number of virus adsorbed on the cell, and occurs with ghosts as efficiently as with intact erythrocytes. HVJ causes some modification of the target cell membrane and enhances phospholipid transfer from the envelope to erythrocyte membrane through the modification. In ghosts, the modification is localized and no enhancement occurs. A model was put forward for the enhancement based on cell swelling; envelope fusion causes an increase in the permeability of target cell membrane and results in cell swelling. The swelling causes modification of the cell membrane which is responsible for the enhancement.

Membrane fusion is associated with many important cellular functions such as endocytosis, secretion of synthesized products from cells, and bulk transport [see Poste & Nicolson (1978)]. HVJ,[1] synonym of Sendai virus, has been used as a simple and reproducible system for the study of fusion mechanisms under controlled conditions, and much information has been accumulated [see Ishida & Homma (1978)]. The enveloped virus has two kinds of membrane glycoproteins, called HANA and F. HANA is responsible for hemagglutinating and neuraminidase activities, while F is essential for hemolytic and fusion activities. The virus first binds to the cell surface, causing gigantic aggregates of cells. The viral envelope then fuses to the target cell membrane, followed by hemolysis and finally cell fusion [see Poste & Nicolson (1977)]. Interrelationships between envelope fusion, hemolysis, and cell fusion are not yet fully understood. Biophysical methods should be able to give detailed molecular insights into the fusion process.

† From the Department of Biophysics, Faculty of Science (T.M., K.K., and S.O.), and Department of Serology and Immunology, Institute for Virus Research (S.T.), Kyoto University, Kyoto, Japan 606. *Received August 14, 1980.* This work was supported by Grant-in-Aid No. 411304 for Special Project Research, Biomembranes, The Ministry of Education, Science and Culture, Japan.
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[1] Abbreviations used: HVJ, hemagglutinating virus of Japan; HAU, hemagglutinating unit; tempocholine, *N,N*-dimethyl-*N*-(2,2,6,6-tetramethyl-4-piperidinyl)-*N*-(2-hydroxyethyl)ammonium chloride; Tris, tris(hydroxymethyl)aminomethane.

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