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119. Hiroshi Ueki,*1 Seigorō Hayashi, and Yosoji Ito*2 : Influence of Quinone Derivatives on Insulin, Glucagon, and Insulinase. IV.[1] Interaction of Insulin with Quinone.

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In the previous paper, it was shown that concurrent or mixed use of quinone monoxime methyl ether (QM) with insulin or protaminezinc insulin prolonged the duration and enhanced the activity of insulin, but not *p*-benzoquinone (BQ), while both QM and BQ markedly inhibited the activity of glucagon and insulinase.

Binding of protein with quinones has been demonstrated in the studies on antibacterial activity of quinones,[2] and the isolation of amino acid-quinone complex[3] and the binding of sulfhydryl group of some amino acids with quinones[4] have been reported. However, the rate and the site of binding have not sufficiently been elucidated.

Many workers discussed the binding of proteins such as bovine serum albumin and insulin with inorganic ions,[5] methyl orange,[6] or phenol red,[7] and reported that some kind of metals such as zinc and silver[8] had some effect on the biological activity of insulin, but interaction of insuline with quinones has not yet been described.

Hitherto, the rate of binding of insulin with small molecular compound was measured by the differential dialysis technique, using a cellophane bag,[9] but insulin may escape from the cellophane bag on account of its small molecular weight. Furthermore, salt solution of higher concentration was used to decrease the influence of the Donnan effect as far as possible, so that a competition between the salt and the substance to bind with insulin was presumed.

To eliminate these disadvantages, chromatographic technique with dextran gel column[10] was used to separate free quinones from insulin-bound quinones, and the rate of binding was determined by spectrophotometer.

The present paper describes the results of some physicochemical procedure for the mechanism of biological action of quinone derivatives.

Experimental

Materials——Crystalline bovine insulin of 23 units/mg.*3 was used, which was found to be almost homogeneous by both ultracentrifugal and electrophoretic analyses.

p-Benzoquinone monoxime methyl ether (QM) and *p*-benzoquinone (BQ) were synthesized in this laboratory.

Determination of the Rate of Binding——Binding of 0.01 m*M* insulin with various concentrations of quinone was carried out in the medium of 0.001*N* HCl of pH 3.0 at room temperature. Immediately

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*3 We are deeply indebted to Shimizu Seiyaku Mfg. Co. for kindly supplying the crystalline insulin.
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2) E. A. Cooper : Biochem. J., **22**, 317 (1928).
3) E. Fischer : Ber., **43**, 525 (1910).
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5) E. Fredericq : J. Am. Chem. Soc., **72**, 2684 (1950).
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after mixing, isolation of free quinones was effected through dextran gel column chromatography, as shown in Fig. 1. The amount of BQ or QM was measured with Cary Model 14 spectrophotometer at 245 mμ or 320 mμ, respectively. The amount of quinone bound to insulin was calculated from the decrease of concentration of free quinone in the solution of quinone containing insulin. Insulin bound to quinone was quantitatively measured with the method of Folin and Lowry.[11]

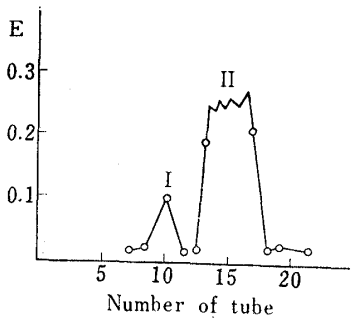


Fig. 1. Chromatographic Separation of free Quinone and Insulin Bound to Quinone with Dextran Gel

dextran gel type : G-25 coarse
column : 12×150 mm.
flow rate : 0.5 ml./min.
solvent : distilled water
I : insulin bound to quinone
II : free quinone

Sedimentation——Sedimentation experiments were carried out after storage, in aseptic state at 1° for 2 weeks, a mixture of 0.8% insulin and 0.1% quinones dissolved in 0.001*N* HCl of pH 3.0, by using a Spinco Model E ultracentrifuge. The runs were made at a speed of 59,780 r.p.m. at room temperature.

Electrophoresis——The mixture described above was dialyzed for 24 hr. against pH 3.6 acetate buffer containing 0.1*M* KCl ($\mu=0.3$) and was analyzed electrophoretically by application of the Tiselius techniques.[12]

Paper Electrophoresis——With the same mixture described above, paper electrophoretic analyses were carried out on the Tōyo Roshi No. 51 filter paper (12.5×26 cm.) using barbital buffer (pH 8.6, $\mu=0.05$) and citrate buffer (pH 3.6, $\mu=0.2$) solutions.

After coloring with bromophenol bule, the filter paper was fixed and made transparent with paraffin. The optical density of the colored electrophoretic patterns was measured by a densitometer, with a green filter.

Results

Rate of Binding with Quinones——This experiment, as shown in Fig. 2, was carried out within the range of 0.1 to 1 m*M* concentration of quinone and 0.01 m*M* concentration of insulin. The larger the amount of free quinone, the higher the rate of binding was.

When the concentration of free BQ was 0.36, 0.41, and 0.56 m*M*, the amount of binding of BQ per one molecule of insulin was 1.5, 2, and 7 respectively. When the concentration of f ree QM was 0.23, 0.45, and 0.9, QM bound per one molecule of insulin was 1, 2, and 10, respectively.

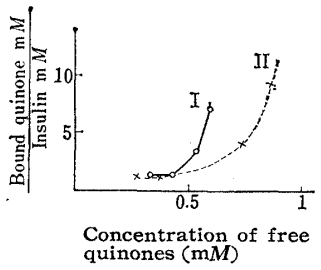


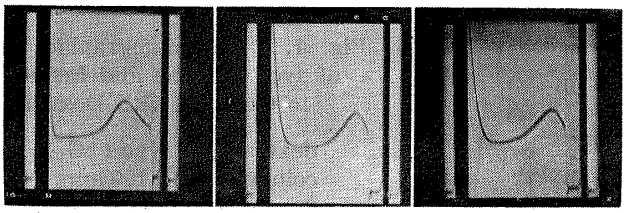
Fig. 2. Rate of Binding of Quinones in Series of Solutions of Various Concentrations of Quinones, immediately after Mixing at Room Temperature

I: BQ, II: QM. concentrations of insulin; 0.01 m*M*, in 0.001*N* HCl of pH 3.0

Sedimentation——Three sedimentation experiments, as shown in Fig. 3, were carried out with insulin, insulin+BQ, and insulin+QM. The measured values of the sedimentation coefficients were corrected to the temperature of 20° in a solvent, with the viscosity and density of water by the usual procedure. The corrected value of the sedimentation

11) O.H. Lowry : J. Biol. Chem., **193**, 265 (1951).
12) J. Hall : *Ibid.*, **139**, 175 (1941).

coefficient was expressed as $s_{20,w}$ and shown in Table I. The value of $s_{20,w}$ was found to be 1.14 on insulin, 1.20 on insulin+BQ, and 1.23 on insulin+QM. The value of the latter in two kinds of samples was larger than that of insulin. From this result, it is assumed that quinone derivatives have a physicochemical effect on insulin molecule.



a b c

Fig. 3. Sedimentation Boundary of Insulin, Insulin+BQ, and Insulin+QM

Sedimentation patterns of a mixture of 0.8% insulin and 0.1% quinones dissolved in 0.001*N* HCl of pH 3.0, obtained with a Spinco Model E ultracentrifuge at 59,780 r.p.m., after storage in aseptic state at 1° for 2 weeks. The direction of centrifugal force is from right to left

a: insulin b: insulin+BQ c: insulin+QM

Table I.

	$s_{20,w}$		$s_{20,w}$
Insulin	1.14	Insulin+QM	1.23
Insulin+BQ	1.20		

Sedimentation Coefficients, $s_{20,w}$ obtained by Patterns shown in Fig. 3

Electrophoresis——As shown in Fig. 4, no difference was observed among insulin, insulin+BQ and insulin+QM, both in the ascending and descending patterns after 10 minutes. However, the migration distance in the pattern of insulin+QM was slightly shorter than those of the other two.

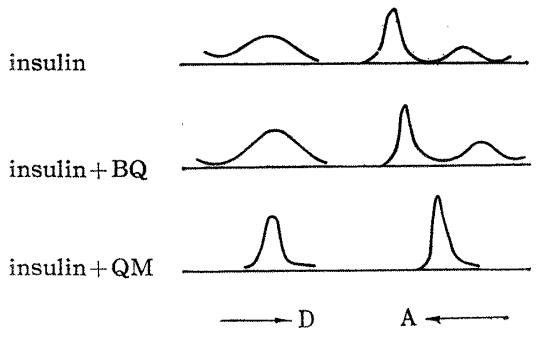


Fig. 4. Electrophoretic Patterns of Insulin, Insulin+BQ, and Insulin+QM

Electrophoretic patterns of a mixture of 0.8% insulin and 0.1% quinones dissolved in 0.001*N* HCl of pH 3.0, obtained after storage in aseptic state at 1° for 2 weeks. Prior to these experiments, the solutions were dialyzed against acetate buffer (pH 3.6) with 0.1*M* KCl (μ=0.3). Acetate buffer in 0.1*M* KCl; ionic strength, 0.3; pH 3.6; 3.4 mA; 130 v.; time, 10 min.; temperature, 4°.

Paper Electrophoresis——When barbital buffer (pH 8.6, μ=0.05) was used, the patterns shown in Fig. 5a were obtained for insulin, insulin+BQ, and insulin+QM. Each showed a small pattern which did not move from the original line, and a large pattern which moved rapidly. The distances of large patterns of insulin+BQ and insulin+QM moved was slightly shorter than that of insulin. With the citrate buffer (pH 3.6, μ= 0.2), as shown in Fig. 5b, insulin showed two patterns, but insulin+BQ and insulin+QM showed only one pattern and the slowly moving pattern of insulin was not observed.

Discussion

When the ratio of concentration of insulin to quinones was below 1:20, binding of insulin with quinones took place slightly and the rate of binding increased as the ratio

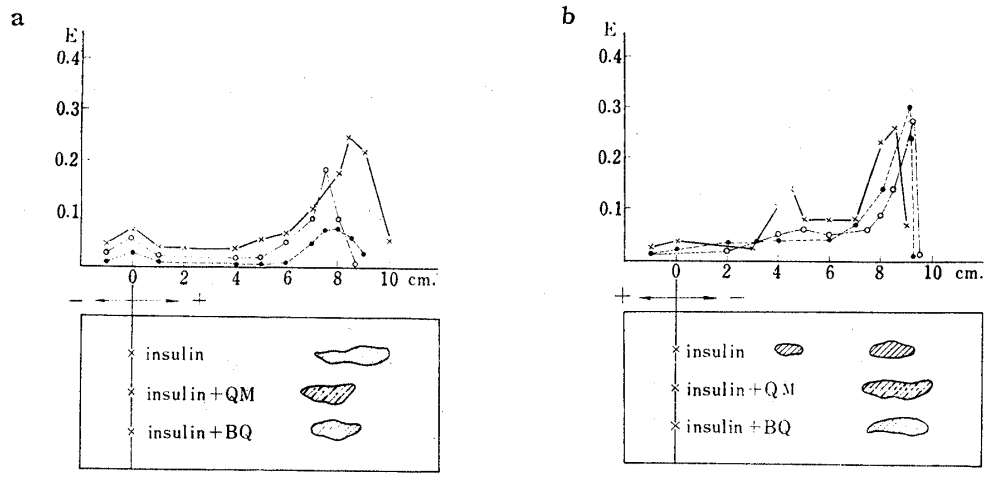


Fig. 5. Diagrams of Paper Electrophoresis of Insulin, Insulin+BQ, and Insulin+QM

Paper electrophoretic patterns of a mixture of 0.8% insulin and 0.1% quinones dissolved in 0.001*N* HCl of pH 3.0, obtained after storage in aseptic state at 1° for 2 weeks

a : veronal buffer; pH 8.6, $\mu=0.05$, 7 mA., 8 hr., bromophenol blue stained
b : citrate buffer; pH 3.6, $\mu=0.2$, 7 mA., 7 hr., bromophenol blue stained

of concentration decreased. In particular, rapid increase was observed at the ratio of 1:50 of BQ and 1:100 of QM. This result shows that the interaction of insulin with quinones depends on the concentration of quinone and that the reactivity of BQ is larger than QM.

In sedimentation analysis, the increase of sedimentation coefficient of insulin on adding quinones suggests that quinones produced some change in molecular form, promoted the association of molecules, or decreased hydration of insulin molecules.

No differences were found among the electrophoretic patterns of insulin, insulin+BQ, and insulin+QM.

In paper electrophoretic analysis, the patterns obtained from each sample in veronal buffer did not show any differences, but migration distance of insulin+BQ and insulin+QM was shorter than that of insulin, and in citrate buffer insulin showed two patterns but insulin+BQ and insulin+QM showed only one pattern.

From these paper electrophoretic analysis, in particular, disappearance of one in the two patterns, it was thought that quinones might produce larger compound than a monomer, dimer or polymer, which exist together in insulin solution.

It was concluded that the direct binding of insulin with quinones, some change in molecular form of insulin, and increase of association of insulin molecules by quinones might give some effect on the biological activity of insulin.

The authors wish to express their thanks to Dr. Y. Kubota and Miss M. Takahashi for sedimentation analysis.

Summary

The binding of insulin with quinones and its derivatives was quantitatively measured by chromatography on dextran gel column and it was found that the rate of binding increased with increasing concentration of free quinones in the range of 0.1 to 1 m*M* of quinones. The binding of quinones with insulin was also studied by using sedimentation, electrophoretic, and paper electrophoretic analysis.

From these results, it was assumed that quinone derivatives might bind to insulin, produce some change in molecular form of insulin, and promote the association of insulin molecule.

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