[Chem. Pharm. Bull. 19(2) 286—291 (1971)]

UDC 547.458.6.09

Studies on Hydroxyethyl Starch as a Plasma Expander. II.[1] Influences of Molecular Weight of Hydroxyethyl Starch on Its Physicochemical and Biological Properties

Terumi Tamada, Kodo Okada, Ryozo Ishida, Katsuyuki Kamishita, and Tsutomu Irikura

Kyorin Chemical Laboratory[2]

(Received June 24, 1970)

Hydroxyethyl starch (HES) was studied concerning the relationships between its physicochemical properties and biological activities to obtain the most desirable plasma expander. Since degree of substitution (DS) had been clarified to have influences on the biological activities, the molecular weight was then examined with designing DS in the constant range of 0.43—0.55. After infusion of 15 ml/kg of 6% HES solution in saline into rabbits, persistence of polysaccharides in blood was determined. HES with higher molecular weight was more persistent when DS was identical. The molecular weight, however, had little influences on the amount of formed reducing sugars when resistance against pig pancreas α-amylase was tested *in vitro*. HES with DS 0.54 and molecular weight about 216000 was hydrolyzed with various concentrations of HCl, and the physicochemical properties and the biological activities of the hydrolysates were also examined. Comparing the lowering tendency of the viscosity with the amount of formed reducing sugar and from the gel filtration patterns of the hydrolysates, it was assumed that the hydrolysis of HES with HCl resulted in separation to two or more intermediate lower molecular weight polysaccharides besides the reducing sugar liberation. Mode of hydrolysis was partly discussed in connection with stability of HES in blood.

Hydroxyethyl starch (HES), one of starch derivatives having several hydroxyethyl groups as substituents, have been studied as a plasma expander. But, the relationships between physicochemical properties and biological activities of HES have not yet been completely established.

In the previous report,[1] degree of substitution (DS) of HES was examined, and it was found that the persistence in blood following intravenous infusion of HES solution into rabbits was increased when DS was high. The resistance against pig pancreas α-amylase *in vitro* was also markedly influenced by DS.

In the previous report,[1] the intrinsic viscosity and the molecular weight of biologically active HES were in the constant ranges, that is, the intrinsic viscosity was in the range of 0.17 dl/g to 0.20 dl/g and the molecular weight was 100000 to 130000. Then, it was thought to be necessary to examine the influences of the intrinsic viscosity and the molecular weight on the biological activities of HES. This paper deals with the influences of molecular weight on the persistence of HES in blood and on the amylase resistance. Further, HES with DS 0.54 and molecular weight about 216000 was hydrolyzed with HCl, and the physicochemical properties and biological activities of the hydrolysates were examined.

Experimental

Materials——HES listed in Table I were obtained from Ajinomoto Co., Ltd. Hydrolysates of HES were obtained according to following procedures. 60 g of HES (DS 0.54 and m.w. 216000) was dissolved in distilled water (about 400 ml), and 1N HCl (5, 10, 25, 50 or 100 ml) was added to the solution. Total

1) Part I: T. Tamada, K. Okada, R. Ishida, and T. Irikura, *Oyo Yakuri*, 4, 505 (1970).
2) Location: *1-3, Ukima, Kita-ku, Tokyo.*

volume was made up to 600 ml with additional water. The reaction mixture in a sealed flask was heated on a water bath at 80° for one hr and neutralized by careful addition of 1N NaOH after cooling. The solution containing hydrolysates of HES was frozen and dried *in vacuo*.

Methods——The average molecular weight of HES was determined by Archibald method.[3] HES (1 g) was dissolved in 100 ml of distilled water, and the solution was centrifuged at 20±0.1° at 6000 rpm for 3 hr in a Hitachi UCA-1A type ultracentrifuge. Relative viscosities of various concentrations of the HES solution were determined using Ubbelohde type viscometer. Reduced viscosities were obtained from the relative viscosities, and these were plotted for the calculation of the intrinsic viscosity from the infinite dilution value.[4] DS was determined according to Lortz.[5]

Gel filtration on Sephadex G-75 was performed on a 40×1.5 cm column. Water was passed through the column for 24 hr prior to use. 2 mg of the hydrolysates of HES (freeze-dried material) was applied to the column. Eluant was distilled water with flow rate of 0.1 ml/min. Total carbohydrates content in each fraction (2 ml) was determined using anthrone.[6]

The HCl hydrolysates of HES were dialyzed in a bag of cellulose casing (Visking) against distilled water. The outside fluid was desalted by the ion-exchange chromatography of Amberlite IRC 50 and Amberlite IRA 401 (1:2, v/v). These dialyzed and desalted samples were spotted on thin-layer (0.25 mm layer) plate of Silica gel G (Merck) and developed in a following solvent; *n*-butanol:2,6-lutidine:ethanol:water (10:5:5:2, v/v/v/v). Anisaldehyde spray was used for the detection.

Evaluation of the biological activities of HES was carried out as described in the previous report.[1] 6% HES solution in saline was infused into ear vein of rabbits with 15 ml/kg body weight in five min. Blood was taken immediately after the infusion and then 1, 2, 3, 4 and 6 hr later. Blood polysaccharides concentration was calculated from the total carbohydrates concentration by anthrone and blood sugar concentration by Folin and Wu method,[7] and was expressed as % to the concentration at the end of the infusion.

Resistance to pig pancreas α-amylase *in vitro* was examined as follows. Mixture of substrate (10 mg/ml) and α-amylase (0.5 μg/ml) in 0.02M phosphate buffer (pH 6.9) was incubated at 30°. Formed reducing sugar was determined using 3,5-dinitrosalicylic acid.

Result

Influences of the Molecular Weight on the Properties of HES

The relationship between the intrinsic viscosity and the average molecular weight of HES determined by Archibald method is shown in Fig. 1. Following equation was established from Fig. 1.

$$[\eta]=4.72\times10^{-4}M^{0.517}$$

Thus, increased molecular weight resulted in high intrinsic viscosity.

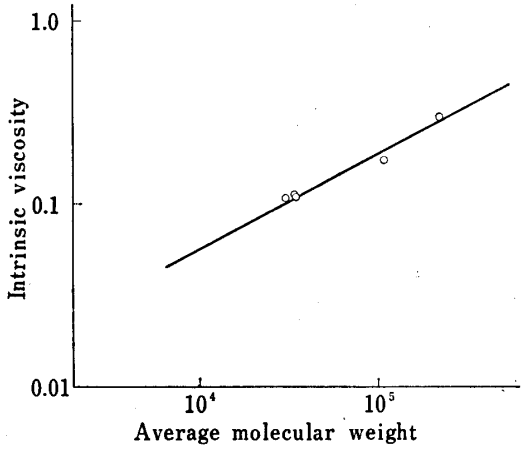

Fig. 1. Relation between Average Molecular Weight and Intrinsic Viscosity of HES

In Table I, physicochemical and biological properties of HES with various molecular weights are shown. When comparing HES, whose DS were in the constant range each other, it was evident that molecular weight had little influence on the resistance against α-amylase. Blood polysaccharides concentrations after intravenous infusion of 6% HES solutions are indicated in Fig. 2. HES F (m.w. 222000) was shown to be more persistent than HES G (m.w. 34000) even though DS were identical. As an exception, HES E (m.w. 147000), which was apt to be hydrolyzed by α-amylase *in vitro*, was less persistent than HES H (m.w. 105000).

3) W.J. Archibald, *J. Phys. Chem.*, **51**, 1204 (1947).
4) E.O. Kraemer, *Ind. Eng. Chem.*, **30**, 1200 (1938).
5) H.J. Lortz, *Anal. Chem.*, **28**, 892 (1956).
6) W.F. Durham, W.L. Bloom, G.T. Lewis, and E.E. Mandel, *Publ. Hlth. Rep., Wash.*, **65**, 670 (1950).
7) O. Folin and H. Wu, *J. Biol. Chem.*, **41**, 367 (1920).

Table I. Influences of Molecular Weight of HES on Its Physicochemical and Biological Properties

Materials	DS	Intrinsic viscosity	Average molecular weight	Hydrolysis	
				Rate[a]	Limit[b]
HES A	0.43	0.200	123000[c]	4.61±0.09	12.7
HES B	0.45	0.104	35000	4.92±0.07	11.7
HES C	0.48	0.287	247000[c]	2.27±0.06	7.6
HES D	0.50	0.300	269000[c]	2.53±0.06	8.2
HES E	0.51	0.220	147000[c]	3.51±0.05	9.9
HES F	0.52	0.290	222000	2.59±0.05	9.8
HES G	0.52	0.108	3400	2.56±0.06	7.2
HES H	0.53	0.184	105000[c]	1.87±0.08	7.8
HES I	0.54	0.103	31000	1.97±0.16	7.7
HES J	0.55	0.168	110000	1.83±0.08	7.2

a) Hydrolysis rate was expressed in terms of 10^{-3} mg of maltose liberated per unit enzyme in one minute; 10^{-3} mg/min/unit. Values were mean ±S.E. of 6 experiments.
b) The proportion of the amount of formed reducing sugar to the amount of complete hydrolysis.
c) Values were calculated from the intrinsic viscosity using the equation in text.

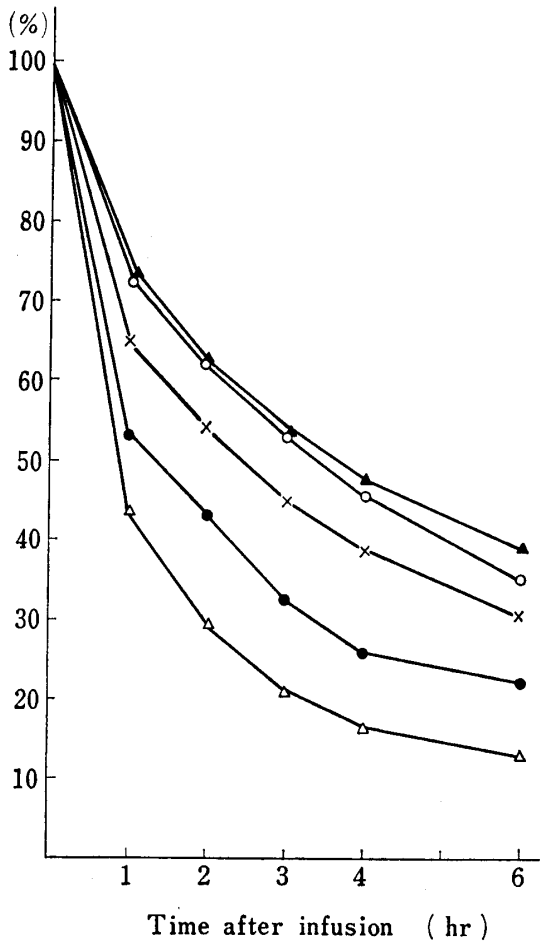

Fig. 2. Blood Polysaccharides Concentration following Infusion of 6% Solution of HES into Rabbits

Blood polysaccharides concentration was expressed as % to the concentration at the end of infusion.
—▲—: HES C —●—: HES E —○—: HES F
—△—: HES G —×—: HES H

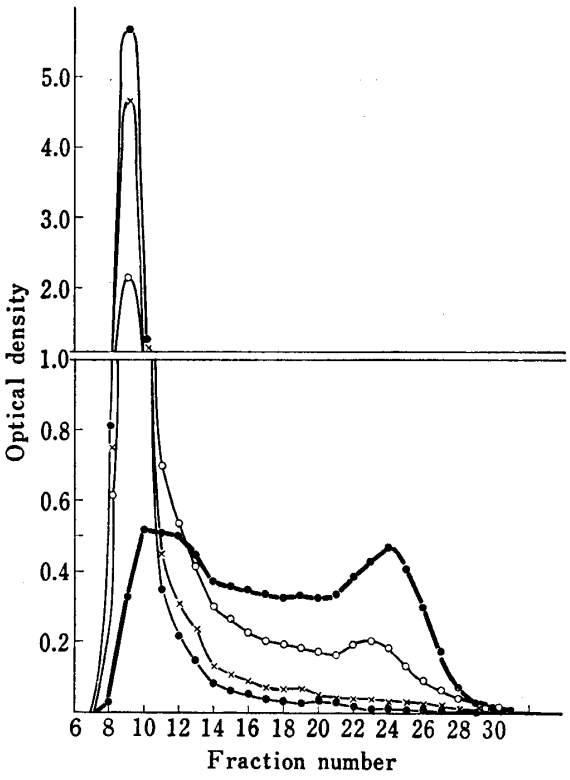

Fig. 3. Gel-filtration of Hydrolysates of HES by Sephadex G-75

—●—: HES 1 —×—: HES 3
—○—: HES 5 —●—: HES 6

Physicochemical and Biological Properties of the Hydrolysates

As shown in Table II, the intrinsic viscosity was lowered with increasing concentration of HCl, and the value of HES hydrolyzed with 1/6N HCl was less than one-third as compared with that of HES before hydrolysis. The amount of formed reducing sugars was increased when the concentration of HCl was high. DS of the total hydrolysates of HES was slightly altered by hydrolysis, but did not show a general tendency.

TABLE II. Physicochemical Properties of HES hydrolyzed with Various Hydrochloric Acid Concentrations

Materials	Concentration of HCl	DS	Intrinsic viscosity	Relative[a] viscosity	Reducing sugar (%)
HES 1	0	0.54	0.286	4.77	0.00
HES 2	1/120N	0.56	0.258	3.93	0.13
HES 3	1/ 60N	0.58	0.209	3.27	0.40
HES 4	1/ 24N	0.52	0.155	2.40	1.15
HES 5	1/ 12N	0.54	0.103	1.92	2.30
HES 6	1/ 6N	0.50	0.067	1.54	6.48

a) relative viscosity of 6% solution

Gel filtration patterns of the hydrolysates of HES in Table II are shown in Fig. 3. With increasing the concentration of HCl, low molecular weight fractions were gradually increased. It was reconfirmed that molecular weight was considerably lowered with increasing the concentration of HCl.

Thin-layer chromatogram of the hydrolysates of HES is shown in Fig. 4. HES before hydrolysis remainea on the spotted point. Hydrolyzing HES with 1/120N HCl, a spot with *Rf* value corresponding to glucose

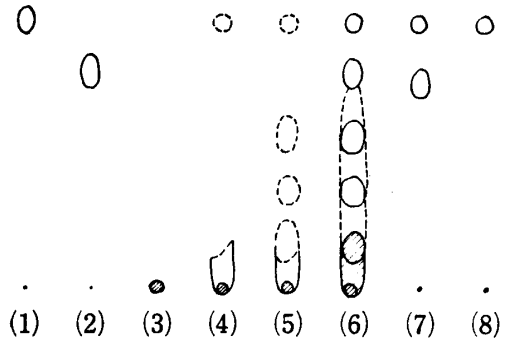

Fig. 4. Thin-Layer Chromatogram of HES hydrolyzed with Various Concentrations of Hydrochloric Acid

Adsorbent: silica gel G; developing solvent, *n*-butanol: 2,6-lutidine:ethanol:water (10:5:5:2) and detected by anisaldehyde spray.
(1) glucose, (2) maltose, (3) HES 1, (4) HES 2, (5) HES 4, (6) HES 6, (7) HES completely hydrolyzed with 8N HCl for 48 hr, (8) corn starch hydrolyzed with 8N HCl for 48 hr.

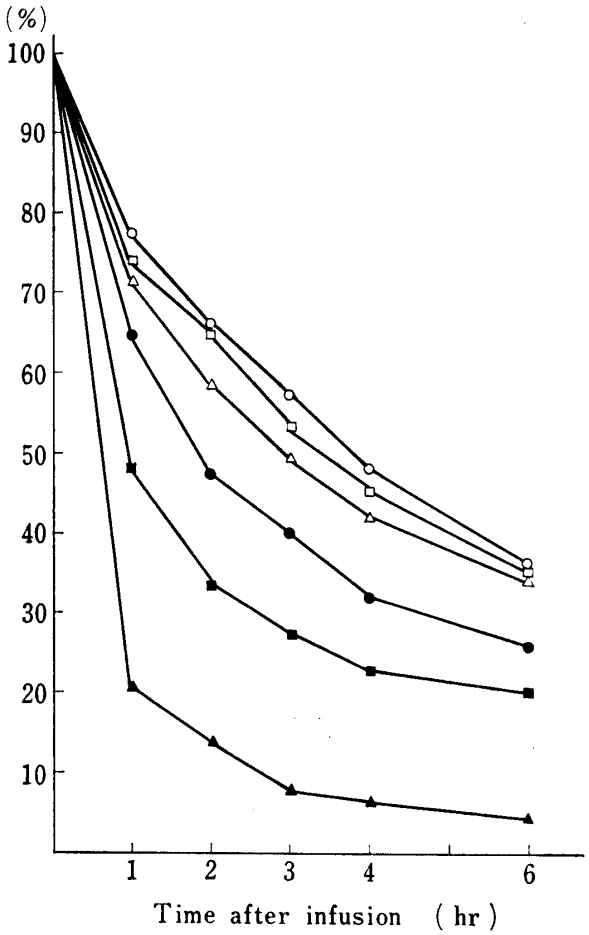

Fig. 5. Blood Polysaccharides Concentration following Infusion of Hydrolysates of HES

Blood polysaccharides concentration was expressed as % to the concentration at the end of infusion.
—○—: HES 1 —●—: HES 4
—□—: HES 2 —■—: HES 5
—△—: HES 3 —▲—: HES 6

was detected in addition to the spot on the spotted point. When HES was hydrolyzed with 1/24N HCl, spots of two partially solvolyzed intermediates were observed under the spot of maltose, and they were intensified on the sample hydrolyzed with 1/6N HCl. Moreover, a spot corresponding to maltose was detected when 1/6N HCl was used. When HES was completely hydrolyzed with 8N HCl at 37° for 48 hr, two spots corresponding to maltose and glucose were detected, respectively. Complete hydrolysis of corn starch, however, resulted in appearance of only one spot of glucose.

Blood polysaccharides concentration following infusion of 6% solution of the hydrolysates is shown in Fig. 5. With increasing the concentration of HCl, the persistence in blood of the hydrolysates was decreased. There was observed some linear relationship between the half life of the hydrolysates in blood and the intrinsic viscosity as shown in Fig. 6.

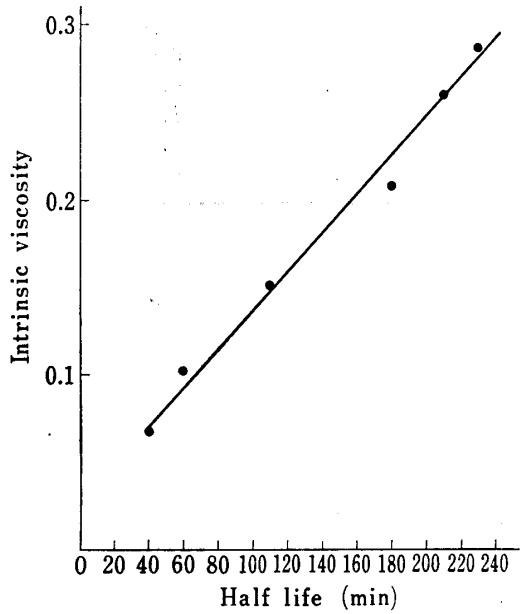

Fig. 6. Relation between Half Life and the Intrinsic Viscosity of Hydrolysates of HES

Half life was a time from the end of infusion of hydrolysates of HES, enough for the disappearance of 50% blood polysaccharides.

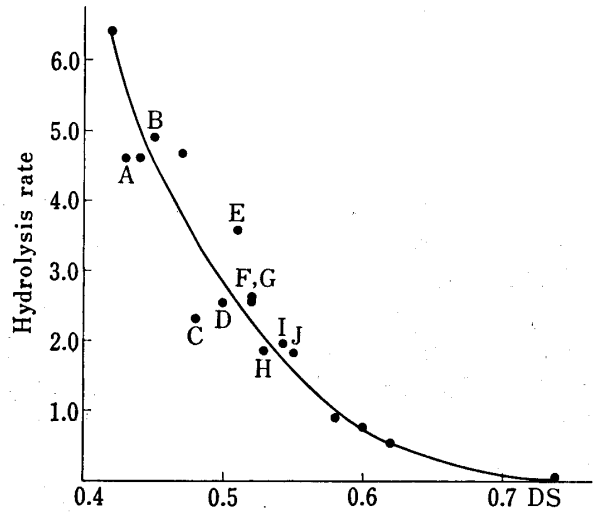

Fig. 7. Relation between DS and Hydrolysis Rate by Pig Pancreas α-Amylase *in Vitro*

A—J means HES A—J for Table I and others for the previous report,[1] respectively.

Discussion

Hydroxyethyl starch (HES), which was first introduced by Wiedersheim[8] as a plasma expander, was more resistant against α-amylase and more persistent in blood than soluble starch. Extensive studies have been made by Thompson and Walton, *et al.*[9] However, the the relationships between physicochemical properties and biological activities of HES have not yet been completely established. HES with DS 0.7 was studied by Thompson, *et al.*[9] in dogs, by Solanke[10] in man, and was reported to be effective as a plasma expander. HES with DS 0.9, 0.85, and 0.8 were studied by Maurer, *et al.*[11] about their possible antigenicity, but were shown to be non-antigenic in experimental animals and in man. HES with DS

8) M. Wiedersheim, *Arch. Intern. Pharmacodyn.*, **111**, 353 (1957).
9) W.L. Thompson, J.J. Briton, and R.P. Walton, *J. Pharmacol. Exptl. Therap.*, **136**, 125 (1962); W.L. Thompson and R.P. Walton, *ibid.*, **143**, 131 (1964); *idem*, *ibid.*, **146**, 359 (1964); W.L. Thompson, D.H. Wayt, and R.P. Walton, *Proc. Soc. Exptl. Biol. Med.*, **115**, 474 (1963).
10) T.F. Solanke, *Brit. Med. J.*, **III**, 783 (1968).
11) P.H. Maurer and B. Berardinelli, *Transfusion*, **8**, 265 (1968).

0.9, however, had some undesirable properties of prolongation of bleeding times in dog.[12] There seems to be few reports concerning HES with lower DS values or clarifying the most appropriate molecular weight as a plasma expander.

In the previous report,[1] we examined the relationships between DS and biological activities of HES and showed that HES with lower DS value under 0.6 was also an expectable plasma expander. Continued from the preceding report, relationship between molecular weight of HES and biological activities was studied with designing DS in the constant range of 0.43——0.55. HES with higher molecular weight seemed to be more persistent in blood. However, as an exception, HES E (m.w. 147000) was shown to be less persistent than HES H (m.w. 105000). Considering the relationships between DS and the persistence, it seems to be incompatible that HES C (DS 0.48 and m.w. 247000) was more persistent than HES F (DS 0.52 and m.w. 222000). These results may be explained by the difference in the resistability against α-amylase. Hydrolysis rate by α-amylase was about 3.51 for HES E, 1.87 for HES H, 2.27 for HES C and 2.59 for HES F, respectively.

As considered from the relationship between DS and the hydrolysis rate, HES C and HES E were deviating from the relation curve shown in Fig. 7. These deviation may be attributed to the difference in the distribution patterns of hydroxyethyl groups in HES molecular. Srivastava, *et al.*[13] examined the distribution pattern of hydroxyethyl groups using commercial HES of DS 0.1 and reported that 84% of hydroxyethyl groups substituted at C-2 position, most of remaining 16% at C-6 and a trace amount at C-3 position of glucose unit. Bollenback, *et al.*[14] examined the distribution using HES of DS 0.6 and reported the distribution ratio of hydroxyethyl groups on the C-2, C-3, and C-6 position as 5.6:0.2:1.0. These distribution patterns of hydroxyethyl groups should have some influences on the resistance against α-amylase. In order to estimate the persistence of HES in blood, it was thought to be necessary to determine not only DS and molecular weight but also the resistance against α-amylase.

In this report, hydrolysates of HES were also examined. The amount of formed reducing sugars was increased when the concentration of HCl was higher. But the increase of the reducing sugars was not so great as considered from the lowering tendency of the viscosity. It is assumed that the hydrolysis of HES with HCl results in separation to two or more intermediate lower molecular polysaccharides besides the reducing sugar liberation.

Number of glucose units having hydroxyethyl substituents among total glucose units of the hydrolysates, as expressed as DS, was not significantly changed. Thus, hydrolysis probably occurs at 1–6 linkage or 1–4 linkage of HES, and cleavage of the ether linkage with release of ethylene glycol should not occur. Bogan, *et al.*[15] observed quite a small amount of $^{14}CO_2$ in expired air following infusion of ^{14}C-labeled HES in animals. He assumed that the cleavage of the ether linkage should not occur *in vivo*. This assumption must be followed in detail to make sure the safety of HES solution as a plasma expander.

Each molecular weight fraction of hydrolysates were separated using Sephadex G-75 gel filtration. Hydrolysis products were also detected on thin layer plate of Silica gel G. Identification of hydrolysates is now under investigation and results will be published in the near furture.

Acknowledgement The authors are deeply indepted to Research Laboratories of Ajinomoto Co., Ltd. for supplying hydroxyethyl starch and determining the degree of substitution. We wish to thank Dr. S. Ogihara, President of Kyorin Pharmaceutical Co., Ltd., for his continuous encouragement. We are also grateful to Mr. K. Shirai and his collaborators for their kind advices.

12) W.L. Thompson and R.H. Gadsden, *Transfusion*, **5**, 440 (1965).
13) H.C. Srivastava and K.V. Ramalingam, *Stärke*, **19**, 295 (1967); H.C. Srivastava, K.V. Ramalingam, and N.M. Doshi, *ibid.*, **21**, 181 (1969).
14) G.N. Bollenback, R.S. Golik, and F.W. Parrish, *Cereal Chem.*, **46**, 304 (1969).
15) R.K. Bogan, G.R. Gale, and R.P. Walton, *Toxicol. Appl. Pharmacol.*, **15**, 206 (1969).