Fate of Butylated Hydroxyanisole in Man and Dog

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To see if results in rats, rabbits, and dogs at high dose levels justified extrapolation, the fate of butylated hydroxyanisole in man was studied. Further possible pathways were sought in dogs. Dogs excreted 60% of a 350 mg. per kg. dose unchanged in the feces within 3 days, and the remainder in the urine largely as sulfate conjugates of BHA, *tert*-butylhydroquinone, and an unidentified phenol. Little glucuronide was formed. Procedures used for low dose levels in rats were applied to man, where 0.5 to 0.7 mg. per kg. yields in the urine less than 1% of the dose as unchanged BHA, and 27 to 77% as the glucuronide of BHA, mostly within 24 hours. No dealkylation or hydroxylation was found. Small doses in man and large doses in rats and rabbits have similar fates, and extrapolation from these species is justified. Comparative metabolism studies are important for safety evaluation of additives, particularly if extension to man is permissible.

Butylated hydroxyanisole, BHA, a mixture of more than 96% of 3- and less than 4% of 2-*tert*-butyl-4-methoxyphenols, was introduced as a fat stabilizer about ten years ago. It has found general use in a variety of products, and its use as an additive in foods has been accompanied by a number of studies of its safety. Acute and chronic studies have shown BHA to have a low toxicity in dogs (*6*, *9*) and rats (*8*). No adverse effects on humans have been reported. Prolonged dosage of rats at several diet levels caused no detectable storage in the perirenal, omental, or subcutaneous fat (*8*).

The metabolic fate of several antioxidants, including BHA, has recently been reviewed (*4*), and studies on the fate of BHA in the rabbit (*5*), dog (*9*), and rat (*1*) have been reported. In the rabbit, an oral 0.5-gram dose was excreted as glucuronide (60%), ethereal sulfate (12%), and uncombined phenols (4%), the constituent isomers being metabolized similarly. In the dog, elevated glucuronic acid output and ethereal sulfate ratios accompanied repeated daily doses of 0.25 gram per kg. In the rat, single doses of 0.4 gram per kg. were accounted for as glucuronide (61 to 82%), ethereal sulfate (11 to 16%), and unchanged BHA (5%). The 3-*tert*-butyl isomer underwent conversion mostly to its glucuronide in the rat, whereas the 2-*tert*-butyl isomer formed mostly a sulfate, and a small proportion seemed to be demethylated (*1*).

No metabolic studies of BHA in man have been reported. Extrapolation to man from animal studies appears to involve several unknowns. Species differences involve differences in metabolic pathways. The dosages used in these studies are large compared with the estimated human intake, which is probably less than 0.1 mg. per kg. Large doses in experimental studies generally produce stress clearly absent with somewhat smaller doses and may also cause the use of metabolic pathways unused with small doses. The order of ethereal sulfate conjugation varies with dose level (*3*), so that the relative proportions of metabolites formed may vary with the size of the dose. A similar result, involving in addition any unmetabolized fraction of the dose, may follow where any of the rates of conjugation, excretion, and renal tubular secretion or excretion are related to the dose level. Therefore, information about the metabolism of a substance gained from large dose studies may be misleading.

We have reported a study of variation in metabolic pathway with dose in the rat (*1*). At dose levels of from 100 to 2 mg. per kg., a fairly close correspondence with metabolism at the large dose level was found, with 81 to 100% recovery of the dose. At low dose levels the proportion of the dose excreted unaltered in the urine increased somewhat, possibly because rates of excretion and conjugation became comparable. The time required for complete excretion of the dose decreased as the dose decreased, and the time for glucuronide excretion at the lowest level was of the same order as that for the unchanged BHA.

Since the ultimate use of BHA is in man, we judged it essential to apply the procedures developed with rats to man, and report the results in this paper. As a guide to the detection in man of further possible metabolic pathways, the fate of BHA in dogs was also studied.

Procedures

Dosages, Diets, Methods. Dogs. Three male mongrel dogs of about 14-kg. weight, fed Purina Dog Chow, were kept singly in metabolism cages. Some difficulty was encountered in feeding large single doses of BHA, but doses of about 5.0 grams (0.35 gram per kg.) were given as 7.5% (w./w.) mixtures in lard, subsequently mixed with the daily diet. Urines were collected after dosing until no further metabolites were detectable; feces were collected for the same period and pooled for each dog. Control values for metabolites were established by analysis of urines collected for a few days before dosing. Excreta were analyzed immediately or kept frozen until analysis.

Humans. Male adult volunteers from this department received single oral doses of BHA, given as the crystalline powder (50 mg.) in a gelatin capsule, or as a homogenate in milk–olive oil (100 to 1 by volume). After mixing, the homogenate was decanted and sufficient ingested to give about a 30-mg. dose. A sample of the material ingested was assayed by continuous ether extraction, followed by spectrophotometric estimation of the ether-extracted BHA (*1*). Urines were collected until no metabolites were detectable. Each voiding was kept separate and where possible refrigerated. Control values for each subject were established before dosing.

BHA was detected after chromatography in the solvent systems (Table I), and was estimated densitometrically, after spraying with Gibbs' reagent (2,6-dichloroquinonechlorimide) and a 2% borate solution, at 595 mμ by the procedure previously described (*1*). Whatman No. 1 filter paper was used throughout in the descending technique.

Examination of Dog Urine. Total and inorganic sulfate content was determined by Folin's gravimetric method (*2*). The glucuronide of BHA was estimated by the enzymic hydrolysis procedure used for low dose studies (*1*), as normal glucuronide excretions were too large and the changes in level too small for the naphthoresorcinol method to be used. Unchanged BHA was determined chromatographically and densitometrically (*1*). Control and experimental urines (10 or 20 μl.) were chromatographed to detect urinary BHA or any chromogenic metabolites (*1*). Solvent systems and spray reagents are listed in Table I.

Examination of Dog Feces. The collection from dosing experiments or an aliquot of control feces was exhaustively extracted (Soxhlet) for 48 hours with benzene. BHA in the benzene extract was identified and estimated chromatographically using Gibbs' reagent (*1*). Attempts to isolate BHA from the extract by steam or vacuum distillation resulted in very impure oils of high BHA content.

Processing of Dog Urine for Metabolites of BHA. Chromatographic examination of daily urines revealed that one, and probably two, chromogenic substances, compounds I and J, absent from control urines, occurred in urines following the ingestion of single large doses of BHA (Table I). The maximum detectable amounts occurred on the first and second days after dosing; by the fourth or fifth day, no metabolites were detectable. Free BHA could not be detected chromatographically in experimental urines.

Urine (2000 ml.) from two dogs, each receiving a 5-gram dose, collected on the three days following the dose, was pooled and the acid and alkaline glucuronide gums were prepared as previously described (*1*) by lead salt precipitation. Both gums contained small amounts of the glucuronide of BHA, as shown by the liberation of BHA by treatment of the gum with β-glucuronidase (*1*). Chromatographic examination showed that the alkaline gum was considerably enriched with two substances absent from a control gum (compounds I and J, Table I); these were considered to be conjugates of metabolites of BHA, but not glucuronides, since they were unaffected by β-glucuronidase.

The possibility that the two metabolites were ethereal sulfates led to the following procedure for their isolation.

The alkaline gum was dissolved in H_2O (100 ml.) and neutralized with solid K_2CO_3. An inorganic precipitate was filtered out and the solution evaporated to a gum. This was repeatedly extracted with warm 90% aqueous (v./v.) 2-propanol (total, 1 liter), followed by hot absolute ethanol (total, 700 ml.) until in each case no further metabolites were extracted. The combined solvents yielded a gum, to the aqueous solution (30 ml.) of which was added safranine bluish (Distillation Products Industries, Rochester, N. Y.) (5.0 gram) in water (100 ml.). The precipitated phenazinium salts of the sulfate conjugates (*1*) were collected the next day, and after aqueous washing yielded a brown-green solid (4.2 grams). This refused crystallization and the phenazinium ion was exchanged for K^+, using Dowex 50 (K^+ form) (*1*). The aqueous solution of potassium salts was evaporated to a small semisolid residue which refused crystallization. Chromatography showed the residue to be largely compounds I and J. It was warmed with 3*N* HCl (20 ml.) for 20 minutes at 60°, cooled, and extracted with ether (3 × 50 ml.).

The aqueous solution gave a strong positive SO_4^{-2} reaction, and the ethereal solution, on drying ($MgSO_4$) and evaporation, gave a small amount of a yellow oil. Chromatography showed this to contain three phenols (A, B, and C, Table I). A was shown chromatographically to be BHA, and B had the constants and reactions of *tert*-butylhydroquinone.

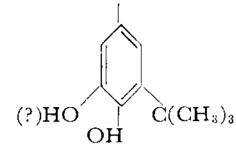
Attempts to crystallize this oil or fractionate it with solvents failed, and it was partitioned on a previously equilibrated column of powdered filter paper (Whatman No. 1) with benzene–acetic acid–water (2:1:1 by volume; organic phase). Fractions of 5 ml. were collected automatically and combined on the basis of maximum absorption at 290 mμ into fractions I (5-ml. fractions 5 to 11), II (13 to 15), and III (41 to 59). BHA and a trace of phenol C were located chromatographically in fraction I, *tert*-butylhydroquinone in II. III contained no metabolites and was discarded.

Fraction II was evaporated in vacuo to a crystalline residue, which on solution in hexane slowly deposited needles

Table I. Chromatography of Ethereal Sulfate Conjugates of BHA and Its Metabolites in the Dog

Solvents
- I. Organic phase of freshly mixed butanol–acetic acid–water (4:1:5 by vol.)
- II. Organic phase of benzene–acetic acid–water (2:2:1 by vol.)

Spray Reagents
- A. Aq. 1% (w./v.) $AgNO_3$ mixed with 1 part aq. 3*N* NH_4OH
- B. Ethanolic 0.05% (w./v.) 2,6-dichloroquinonechlorimide; the dried paper was sprayed with aq. 2% (w./v.) sodium borate, or
- C. With aq. 10% (w./v.) Na_2CO_3
- D. Equal volumes, freshly mixed, of 1% (w./v.) *p*-nitraniline in 3*N* HCl and aq. 5% (w./v.) $NaNO_2$. The dried paper was sprayed with aq. 2% (w./v.) Na_2CO_3.

Source	Substance	Solvent Systems, R_f Values		Colors Given by Reagents[a]				Assigned Structure
		I	II	A	B	C	D	
Urine after 350 mg./kg. dose.	Compound I	0.85	0.00	0	Blue-purple	Blue	Orange	*O*-Sulfate conjugates of phenols B and C
	Compound J	0.75	0.00	0	0	Purple	Red-purple	
Ether extract of hydrolyzate of potassium salts of etheral sulfate conjugates	Phenol A	0.96	0.94	Blue-brown	Blue	Purple	Orange	BHA
	Phenol B	0.96	0.55	Black	Blue-purple	Purple	Orange	*tert*-Butylhydroquinone
	Phenol C	0.96	0.80	Black	Blue-purple	Purple	Orange-red	

[a] With aq. 2% (w./v.) $FeCl_3$ solution, by phenol C only, yellow, turning to emerald green with *N* Na_2CO_3.

(22 mg.) of *tert*-butylhydroquinone (m.p. 126–9°) undepressed on admixture and having ultraviolet and infrared spectra identical with an authentic specimen. Calculated for $C_{10}H_{14}O_2$: C, 72.2; H, 8.5%. Found: C, 71.9; H, 8.4%.

Attempts to isolate phenol C from either fraction I or other hydrolyzates and fractions were unsuccessful and this substance appears to be largely destroyed on attempted separation. The urine remaining after precipitation of the glucuronide fractions was deleaded and found to contain only trace amounts of compounds I and J; it was discarded. Portions of both glucuronide gums were incubated with β-glucuronidase (*1*) and the hydrolyzates extracted with ether. BHA was detected, but no phenols derived from possible conjugates of BHA metabolites were found. Because of the small quantity of BHA glucuronide present, it was not possible to isolate it.

A portion of the ethereal sulfate of the major isomer of BHA, prepared as previously described (*1*), was submitted to the hydrolytic procedure described above. BHA was the only detectable phenolic product, thus excluding the possibility that phenol C and *tert*-butylhydroquinone were artifacts of BHA or its sulfate.

Examination of Human Urines. Experimental and control urines were analyzed for unchanged BHA and its glucuronide by procedures essentially similar to those described for low dose studies in the rat (*1*). The glucuronide estimation was modified to diminish interference from normally occurring phenolic glucuronides as follows.

The enzymic hydrolysis was performed by mixing 15 ml. of 0.2*M* pH 4.8 phosphate buffer, the corresponding glucuronide fraction from 10 ml. of human urine, and 7 ml. of β-glucuronidase solution. After incubation, the mixture was twice extracted with ligroin (50 ml., b.p. 35–60°). The extracts were centrifuged to de-emulsify them and washed with 25 ml. of aqueous 7% (w./v.) $NaHCO_3$, the alkaline extract was washed with ligroin (50 ml.), and the combined ligroin extracts were cautiously evaporated on a steam bath.

In this way phenolic acids having chromatographic properties identical to those of BHA were prevented from interfering with the final densitometric determination of the BHA content of the residue (*1*).

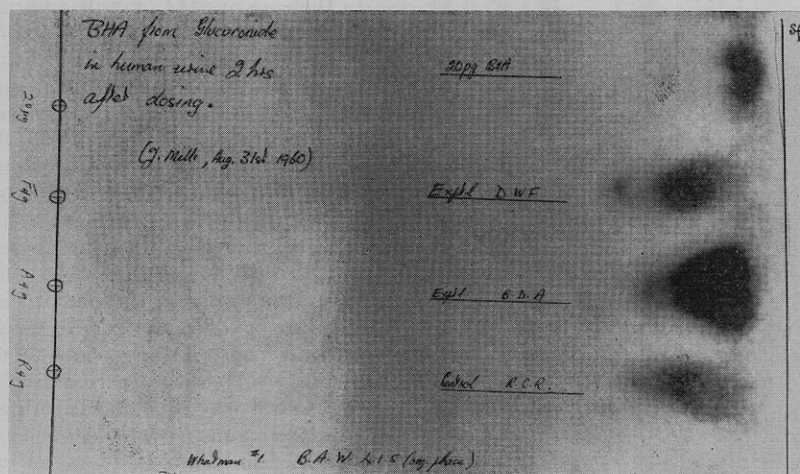


Figure 1. Chromatograms of BHA

Upper. 20 µg. of BHA
Center. Two human subjects each receiving 30 mg. of BHA; spots are BHA derived from its glucuronide
Lower. Human control urine
Spray reagent. B (Table I). BHA spots, blue-purple. Control spot, weak brown-green

Results

The average control values (expressed as BHA) for daily excretions by the three dogs were: free phenols (six determinations) 1.9 (1.0 to 2.3) mg.; glucuronides (six determinations by enzymic procedure) 5.2 (3.8 to 6.0) mg.; ethereal sulfates (14 values) 0.32 (0.2 to 0.49) gram, ethereal–inorganic sulfate = 0.230. Normal daily excretions by humans, in 12 determinations on eight subjects, as BHA were: free <1mg.; of the glucuronide of BHA, 4.0 (2.5 to 5.5) mg.

A study with 350 mg. per kg. doses in dogs showed that most of the dose was readily recovered from the excreta, but several differences from the metabolism pathway in rats and rabbits were found (Table II). A large proportion of the dose is excreted by the dog unaltered in the feces. Glucuronide conjugation is not the main metabolic pathway, but a preponderant sulfate conjugation of the absorbed material is found. Separation of the sulfate conjugate by the phenazinium salt method used for the rat and subsequent ion exchange and mild hydrolysis of the potassium salts showed the elevated sulfate level to be caused partly by the formation of the 4-*O*-sulfate of BHA and two further sulfate conjugates. The isolation of *tert*-butylhydroquinone, derived from one of these, establishes *O*-demethylation as a significant metabolic pathway for BHA in the dog. The other conjugate yielded a phenolic substance whose color reactions on paper chromatograms are consistent with its being a nuclear hydroxylation product of BHA. About the same proportion of the dose is excreted in the urine unchanged as in the rabbit and rat, and the glucuronide conjugate, as in those species, appears to be exclusively with the 4-hydroxyl of BHA.

Table II. Excretion of BHA by Dogs

(Mean % recoveries of a 350 mg. per kg. dose, with ranges in parentheses)

BHA	Days after Dosing: 1	2	3	4	Totals
Urinary					
Free	1.1(0.7–1.6)	2.3(1.2–3.0)	0.2(0–2)	0	3.6
Ethereal sulfate	9(1–20)	11(8–18)	2(0–3)	1(0–4)	23.0
Glucuronide[a]	4.0(1.8–8.1)	1.2(0.6–1.8)	0.3(0.2–0.4)	0	5.5
Fecal					
Unchanged[b]					51, 62

[a] Enzymic method (*1*) for small doses.
[b] Determined on 2 dogs.

Table III. Urinary Excretion of BHA by Man

(Free BHA was invariably less than 1% of dose)

Medium	Average Dose, Mg.	Subject	BHA as Glucuronide, % Recovery	Period for Excretion of Glucuronide, Hours
Olive oil–milk homogenate	31	A	77	23
		B	51	23
		C	39	31
		B	36	27
Capsule	50	D	71	23
		B	41	50
		E	27	38
		F	32	27

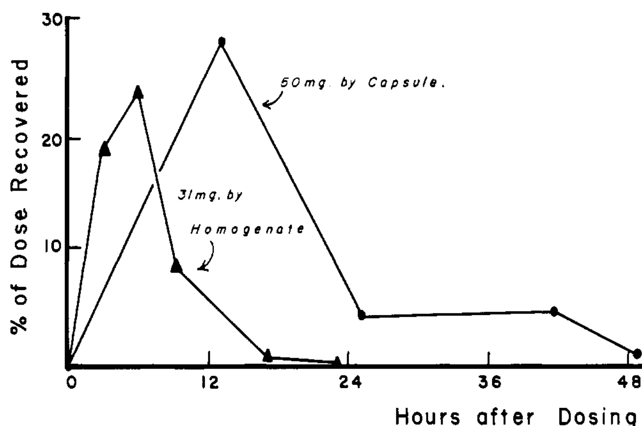


Figure 2. Urinary excretion of BHA as a glucuronide by man

Urines voided at times indicated after dosing.
● slowest and ▲ most rapid excretions of BHA glucuronide at dose levels shown

Table IV. Metabolism of BHA

(% of single dose given)

Species	*Rabbit (5)*	*Rat (1)*		*Dog*	*Man*
Dose, mg./kg.	500	400	2	350	0.4–0.7
Unabsorbed		0–3		56	?
Unconjugated	4	5	10.3	3.6	<1
Ethereal SO_4	12	14		23	Yes (?)
Glucuronide	60	72	72	5.5	27–77
Demethylated		Yes[a]		Yes	No
Hydroxylated		No		Yes (?)	No

[a] Minor metabolite.

In applying the procedures for low doses in rats to man, we found that recoveries of added free BHA from human urine were 90 ± 10%, and of the glucuronide of BHA, less satisfactory than in the rat, 50 ± 7%. BHA in human urine was detectable at concentrations of 1 μg. per ml., and the background absorption on chromatograms, caused by naturally occurring chromogens similar in properties to BHA, was less than 1 μg. per ml. The normally occurring phenolic glucuronides in human urine yielded on enzymic hydrolysis a variety of phenols and phenolic acids. Some of these gave background values on chromatograms where BHA was located of the same order as that expected to be given by BHA at a dose level of 50 mg. A variety of solvent systems was tried without effectively reducing this value. However, the use of ligroin as an extracting solvent, and especially the removal of phenolic acids from the ligroin extract of the hydrolyzate with an alkaline wash, diminished the background.

The average background value for normal phenols and for the glucuronide excretion recorded in Table III was taken as 8 ± 3% of a 50 mg. per kg. dose as BHA. At the levels of dosing recorded, the limits of specificity and precision have been reached in the glucuronide estimation. The lower limit of detectability of the glucuronide was of the order of 2 μg. per ml. of urine.

Table III records the results of analyses of urine from volunteers receiving BHA in each form. Figure 1 shows a chromatogram in which a standard quantity (20 μg). of BHA, BHA derived from its glucuronide in two experimental urines within a short while of dosing, and a control hydrolyzate, are co-chromatographed in solvent I, Table I. Small discrepancies in R_f values of BHA in experimental urines are usual in solvent I, and may be caused by varying urinary constituents. BHA undoubtedly forms a glucuronide in man and, by analogy, probably with its phenolic hydroxyl. Subject to the limits discussed above, 27 to 77% of the dose is excreted in the urine in this form. Urines were also examined for other metabolic pathways, but no demethylated or hydroxylated products could be detected. The possibility of ethereal sulfate formation exists, as dilute acid hydrolysis of some experimental urines occasionally liberated BHA. Free BHA was not detectable in human urines and no account could be made of the remaining nonconjugated BHA. However, absorbed BHA is rapidly metabolized, as Figure 2 shows, where the rates of fastest and slowest excretion for the indicated doses of BHA as a glucuronide are recorded. All other rates of excretion lie within these values. Maximum excretion usually occurs within 17 hours of the dose, and excretion of glucuronide is complete by the second day after ingestion. Refrigeration and immediate analysis, when possible, of collections in the human study were important in obtaining high BHA glucuronide values. Where analysis had to be delayed, glucuronide values were frequently low.

Discussion and Conclusions

As an adjunct to toxicity testing in the safety evaluation of an additive, knowledge of metabolic pathways can be of particular value. Thus, once the existence of absorption has been shown, retention periods within the organism, the likelihood of tissue storage, and whether the absorbed material is converted to normally present metabolites or is metabolized in a fashion usual for the class of compound studied can be ascertained. However, since such results usually result from animal studies only, there is no guarantee of their applicability to man.

In the case of BHA, the theoretically possible metabolic pathways after absorption include conjugation at the phenolic hydroxyl, nuclear hydroxylation and *O*-demethylation, oxidation of the side chain $C(CH_3)_3 \rightarrow C(CH_3)_2.CH_2OH$, and conjugation of the metabolites. Table IV presents the metabolic fate of BHA for all the species so far reported. Without confirmatory evidence in man, no *a priori* reason existed for extrapolating from the results in any one of these species, and a wider spectrum of species widens the choice of any or all of the postulated pathways. It is gratifying to find that a major metabolic pathway in rats and rabbits is also common to man. Within the limitations of the methods of analysis, the great difference between the dose level, in most of the animal studies and the level in man, does not lead to large discrepancies in the manner in which BHA is metabolized.

In this instance, the choice of the dog as the sole experimental animal would have led to highly misleading results. The high level of nonabsorption would have caused speculation about complete nonabsorption at low levels of dosage, and the major metabolic pathway differs from the other species studied. This study illustrates the value of comparative studies, both in species and at various dose levels, in metabolism studies as a part of safety evaluation. The value is enhanced when analytical techniques permit a study in the subject for which safety is being evaluated—i.e., man—of dose levels near those encountered in use.

Acknowledgment

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ALFALFA HEMICELLULOSE

Constitution of the Hemicellulose of Alfalfa (*Medicago sativa*). Further Studies on the Acidic Components Produced by Hydrolysis

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The constitution of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger) is being investigated in an attempt to ascertain the possible relationship between chemical structure and nutritional value for ruminants. Three aldobiouronic acids, 2-O-α-D-galactopyranosyluronic acid-L-rhamnose, 6-O-β-D-glucopyranosyluronic acid-D-galactose, and 2-O-α-D-glucopyranosyluronic acid-L-xylose, have been obtained by acid hydrolysis of alfalfa hemicellulose, in addition to the five acidic components and five neutral sugars reported previously. The results may be of value in ascertaining the role of hemicelluloses in animal nutrition.

THE HYDROLYSIS of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger) leading to a mixture of neutral and acidic components was described in a previous communication (*7*). The neutral components were shown to be L-arabinose, D-xylose, D-galactose, D-glucose, and L-rhamnose. Of the seven acidic components encountered, five were identified as oxalic acid, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose, and *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose. The two other unidentified acids, which were thought to be galacturonic acid containing aldobiouronic acids, are shown herein to be 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R = H) and 6-*O*-β-D-glucopyranosyluronic acid-D-galactose (IV, R=H). Another acid, 2-*O*-α-D-glucopyranosyluronic acid-D-xylose (VIII, $R=R_1=H$), which brings the total to eight, was derived from an aldotriouronic acid which appears to be *O*-α-D-glucopyranosyluronic acid (1→2)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose (VII), the analog of the previously identified aldotriouronic acid cited above which contained 4-*O*-methyl-D-glucuronic acid.

Experimental

All evaporations were carried out in vacuo at 35° to 45° C. (bath temperature) unless specified otherwise.

The ion exchange resins, Amberlite IR-120 (H^+ form) and Duolite A-4 (OH^- form), were used throughout.

The irrigating solvents used in chromatography were: *A*, *n*-butyl alcohol–acetic acid–water (2:1:1); *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *C*, pyridine–ethyl acetate–water (1:2.5:3.5); *D*, *n*-butyl alcohol–ethyl alcohol–water (4:1:5); and *E*, butanone-water azeotrope.

Separation of Uronic Acid Components. The three uronic acids, 2-*O*-α-D-galactopyranosyluronic acid-L-rhamnose (I, R = H), 6-*O*-β-D-glucopyranosyluronic acid-D-galactose (IV, R = H), and an unknown aldotriouronic acid (?VII), which were separated from the other uronic acid components of alfalfa hemicellulose by cellulose column chromatography as described previously (*7*), had approximately the same R_f value (0.16) using solvent *A*. Examination showed that the aldotriouronic acid could be separated from the mixture of the two aldobiouronic acids by electrophoresis (*2*) using Whatman No. 1 paper, 0.1*M* borate buffer, and *p*-anisidine–phosphoric acid spray reagent. Separation on a preparative scale was carried out as follows: The sirup (0.40 gram) was applied to eight pieces of Whatman No. 3MM paper (5 × 22 inches), and each paper was placed on the electrophoresis apparatus (*2*) for 4 hours using 0.1*M* borate buffer, 600 volts and 30 to 40 ma.

After each of the papers had been dried, a strip was cut from the center and sprayed with the *p*-anisidine–phosphoric acid reagent to locate the position of each component. The components were isolated by extracting the appropriate section of the paper with water, and each solution was passed through the cation exchange resin. The effluent was evaporated to dryness. The residue was then treated for 30 minutes with 0.5% methanolic hydrogen chloride at room temperature after which the solvent was evaporated at room temperature to remove borate (*2*). In this manner an aldotriouronic acid (130 mg.), possibly α-D-glucopyranosyluronic acid-(1→2)-D-xylose-(1→4)-D-xylose (VII), and a mixture, *F*, (180 mg.) consisting of the two aldobiouronic acids (I and IV, R=H) were obtained.