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Application of the Carbazole Reaction to the Estimation of Glucuronic Acid and Glucose in some acidic Polysaccharides and in Urine

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A number of carbohydrates may be detected and estimated by means of a reaction with carbazole in sulphuric acid. The methods used have been summarized by Dische (1955). The present paper describes a procedure for the estimation of mixtures of glucuronic acid, glucose and xylose in pure solution, and of glucuronic acid and glucose in various polysaccharides and in urine. Work is also described indicating that the colours obtained with hexoses and pentoses are due to hydroxymethylfurfural and furfural respectively, and that the colour given by the uronic acids is due to neither of these compounds.

Dische (1947) observed that the addition of water to the coloured complex which is slowly formed after mixing carbazole and glucuronic acid in 87% sulphuric acid caused the colour to change and lighten, whereas the slight colour obtained with glucose and other sugars by the same process was intensified. In the present procedure one reading is taken with an absorptiometer 2·5 hr. after the reactants have been mixed; glucuronic acid gives an intense colour at this stage. Water is added, and two further readings are taken on the following day, one with a yellow–green and one with a violet filter. Glucose gives a more intense colour than glucuronic acid at this stage, with maximum absorption of light in the yellow–green region. Pentoses also give a colour at this stage with maximum absorption in the yellow–green region. The pentose colour is less intense than that given by glucose and other hexoses and has a secondary absorption maximum in the violet region, not shown by the hexose colour; this makes possible the detection and rough estimation of pentoses.

MATERIALS AND METHODS

Carbazole. The product from L. Light and Co. gave no colour with H_2SO_4 at 100°. Solutions (0·2 and 0·3%, w/v) of carbazole were made up in A.R. ethanol (water content less than 2%). Both were renewed within 3 months of preparation.

Standards. The stock solution of glucuronolactone (L. Light and Co.) was 1 mg./ml. and the dilute solution 0·1 mg./ml. in water. Both were stored at 4°. The dilute standard was renewed within 1 month, and the stock within 3 months of preparation. Glucuronolactone was used in preference to glucuronic acid because of the difficulty of isolating the acid in the free state. Conversion of the lactone into the barium salt or into the free acid did not affect significantly the readings obtained in the standard procedure.

Other carbohydrates. Glucose (May and Baker Ltd.), galacturonic acid, glucosamine hydrochloride, galactono-γ-lactone and gluconic-δ-lactone (L. Light and Co.), ascorbic acid, furfural and xylose (British Drug Houses Ltd.), arabinose, lactose, galactose and fructose (Hopkin and Williams Ltd.) were used in the same concentrations as glucuronolactone. Bornyl and menthyl glucuronosides were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), potassium hyaluronate and chondroitin sulphate from L. Light and Co., and oxidized cellulose from Parke, Davis Ltd. 5-Hydroxymethylfurfural was prepared from sucrose by the method of Haworth & Jones (1944). Barium glucuronate and glucuronic acid were prepared from glucuronolactone by the method of Goebel & Babers (1933).

Standard procedure

Dilution. Maximum accuracy is only attained when the concentrations of both glucuronolactone and glucose in the test solution are within the range 10–100 μg./ml. and the combined amount of the two compounds is not more than 170 μg./ml. For estimations on urine (guinea-pig or human) suitable concentrations within this range are usually obtainable by diluting 1 ml. of urine to 20 ml.

Method. For each test, pairs of 1 ml. samples of appropriately diluted test solutions, of dilute carbohydrate standards and of water blanks are set up in 25 ml. Pyrex tubes, which are placed in water at 4° for 5 min. Then 6 ml. of ice-cold H_2SO_4 (A.R., Hopkin and Williams Ltd.) are added rapidly to each tube and mixed. All tubes are heated in boiling water for 20 min. and then transferred to water at room temperature. After 2 min., 0·2 ml. of 0·2% carbazole is mixed with one of the solutions in each pair. All tubes are kept in an oven at 26° for 2·5 hr. Light absorption is then measured in a Hilger Spekker photoelectric colorimeter

(H. 760) with an Ilford 604 spectrum green filter. Each tube with the carbazole reagent is read against the corresponding blank without the reagent.

A volume (6 ml.) of each solution was then added to 1 ml. of water (this quantity is critical) previously placed in another 25 ml. tube and 0·2 ml. of 0·3% carbazole is mixed with the solutions to which 0·2% carbazole was originally added. All tubes are kept at 26° until 24 hr. after the first addition of carbazole reagent. Light absorption is then measured as before, except that readings are taken with Ilford 605 spectrum yellow-green and with Ilford 601 spectrum violet filters.

The intensity of the colour with glucuronolactone reaches a maximum between 2 and 2·5 hr. after addition of the reagent and thereafter slowly decreases. The colour with glucose slowly increases beyond 24 hr., but after this time the increase is largely offset by an increase in the reagent blank reading.

Standard nomogram. Estimations made on standard mixtures of glucuronolactone, glucose and xylose showed that the absorption readings after 2·5 and 24 hr. corresponded with the sum of the readings to be expected from the amounts of the components of the mixture taken. On this basis the nomogram shown in Fig. 1 was constructed empirically (e.g. Brodetsky, 1938). The nomogram was used as long as the readings given by the two standard solutions (estimated together with the test solution) differed from those shown on the nomogram by less than 3%. Standard readings outside the ±3% limit occurred after the glucose, glucuronolactone or carbazole solutions had been kept for some time. Slight alterations in the standard and reagent blank readings were sometimes found in changing from one sample of sulphuric acid or ethanol to another. If the standard readings cannot be brought to values within 3% of the readings shown on the nomogram by renewing the reagents, then it is necessary to prepare a fresh nomogram.

Mixtures containing pentose. From the absorption reading with the violet filter, in addition to the two readings used for the nomogram, a check can be made on the presence or absence of pentose.

The ratio $R = E_V/(0{\cdot}465\,E_G + E_{YG})$ for both glucose and glucuronolactone was 0·201, where E_V, E_G and E_{YG} were the absorption readings (minus the corresponding reagent blank readings) obtained with the violet (24 hr.), green (2·5 hr.) and yellow-green (24 hr.) filters respectively. The corresponding ratio for xylose was 0·781 and for arabinose 0·713. A ratio greater than 0·201 was thus taken to indicate (in the absence of galacturonic acid, for which $R = 0{\cdot}258$) that pentose was probably present in the material being investigated. If xylose is present it can be calculated, if it is assumed that Beer's law applies, that the concentration of xylose is $E_V(R - 0{\cdot}201)/0{\cdot}58\,wR$, where w is a constant which is calculated by substituting in the expression the figures for a standard solution of xylose. The corresponding expression for arabinose is $E_V(R - 0{\cdot}201)/0{\cdot}512\,wR$. The numerical factors in all these expressions were altered by changes in the quality of the carbazole, ethanol and sulphuric acid.

When the concentration of pentose has been calculated by one of these expressions, the absorption values corresponding with this content of the pentose may be subtracted from E_G and E_{YG}, and the corrected values used to obtain glucose and glucuronolactone concentrations from the standard nomogram.

For glucose and galacturonic acid $R_1 = 0{\cdot}197 = E_V/(0{\cdot}784\,E_G + E_{YG})$; expressions for calculating pentose concentration may be derived if galacturonic is the only uronic acid.

RESULTS

Some results with various carbohydrates are shown in Tables 1 and 2.

Absorption spectra of the coloured complexes. Absorption spectra of the coloured complexes obtained in the standard procedure with a number of compounds were measured with the Hilger Uvispek (H. 700) spectrophotometer. The curves for hydroxymethylfurfural and furfural (after 24 hr.) are shown in Fig. 2. The curves from glucose, galactose and fructose (after 24 hr.) had the same shape and $\lambda_{max.}$ at 545 mμ as that from hydroxymethylfur-

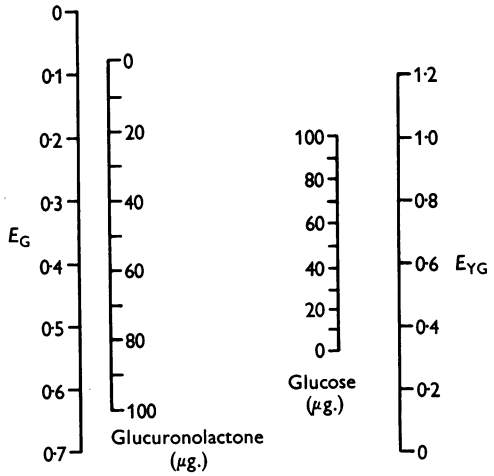

Fig. 1. Nomogram for the estimation of glucuronolactone and glucose from two readings taken with the Spekker absorptiometer; E_G after 2·5 hr., and E_{YG} after 24 hr. by the standard procedure.

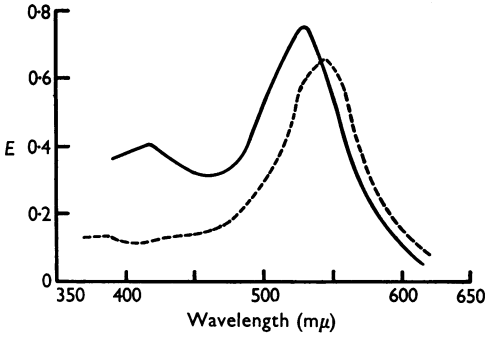

Fig. 2. Absorption spectra of carbazole complexes with hydroxymethylfurfural and furfural treated by the standard procedure. Broken line, 45 μg. of hydroxymethylfurfural; unbroken line, 50 μg. of furfural.

Table 1. *Absorptiometer readings for the coloured complexes obtained by the standard procedure with various compounds*

Substance	Quantity (μmoles)	E at 2·5 hr. Filter 604 (E_G)	E at 24 hr. Filter 605 (E_{YG})	E at 24 hr. Filter 601 (E_V)
Carbazole blank	—	0·007	0·070	0·034
Glucuronic acid	0·568	0·644	0·372	0·152
Galacturonic acid	0·568	0·537	0·375	0·176
Glucuronolactone	0·568	0·650	0·370	0·154
Barium glucuronate	0·568	0·664	0·375	0·157
Bornyl glucuronoside	0·568	0·549	0·320	0·150
Menthol glucuronoside	0·568	0·581	0·312	0·146
Glucose	0·556	0·060	0·940	0·214
Fructose	0·556	0·060	0·960	0·218
Galactose	0·556	0·051	0·640	0·146
Arabinose	0·556	0·020	0·315	0·213
Xylose	0·556	0·024	0·462	0·346
Glucosamine	0·556	0·030	0·096	0·052
Galactono-γ-lactone	0·556	0·009	0·088	0·044
Gluconic-δ-lactone	0·556	0·012	0·087	0·051
Ascorbic acid	0·556	0·012	0·083	0·046
Lactose	0·292	0·050	0·790	0·198
'Soluble' starch	100 μg.	0·051	1·008	0·228

Table 2. *Results obtained by the standard procedure with solutions containing glucuronolactone, glucose and xylose*

In each experiment eight equivalent samples of 1 ml. each were taken for estimation.

Expt. no.	Amount taken (μg.) Glucurono-lactone	Glucose	Xylose	Amount found (mean ± S.D.) (μg.) Glucuronolactone	Glucose	Xylose
1	100	—	—	99·72 ± 1·65	—	—
2	90	10	—	89·44 ± 1·29	10·41 ± 0·91	—
3	50	50	—	50·11 ± 1·28	49·42 ± 1·35	—
4	10	90	—	10·43 ± 0·45	90·67 ± 1·14	—
5	—	100	—	—	99·87 ± 1·46	—
6	50	50	10	49·45 ± 1·03	48·72 ± 0·99	12·67 ± 1·69
7	50	50	50	49·71 ± 0·99	48·93 ± 1·64	48·99 ± 1·29
8	10	10	100	10·37 ± 0·61	11·92 ± 3·04	98·77 ± 2·02

fural. There was a similar correspondence between the curves for xylose and furfural ($\lambda_{max.}$ 530 and 415 mμ). The curve for glucuronolactone after 2·5 hr. had $\lambda_{max.}$ 525 mμ and after 24 hr. 575 mμ.

Determinations on polysaccharides. The percentages (Table 3) of glucuronic acid found in purified hyaluronate, chondroitinsulphate and heparin, and the molar ratio of sulphate:uronic acid in heparin are within the ranges given by previous workers. [For hyaluronic acid, see Roseman, Moses, Ludowieg & Dorfman (1953) and Kent & Whitehouse (1955); for chondroitinsulphate, see Einbinder & Schubert (1950) and Meyer, Linker, Davidson & Weissmann (1953); for heparin, see Foster & Huggard (1955).] The theoretical percentages calculated from the postulated structures of potassium hyaluronate and calcium chondroitinsulphate are 46·1 and 36·6 respectively. The apparent finding of a small amount of glucose in the heparin preparation was not altered by an attempted purification of the preparation by precipitating with cetylpyridinium chloride (see Scott, 1955).

An estimation (not shown in Table 3) on a soluble fraction of gum tragacanth (prepared by the method of Norman, 1931) showed 51·2% by wt. of uronic acid (as galacturonic) and 43·5% of pentose (as arabinose). The whole gum, on the other hand, showed only 34·8% of galacturonic acid, about 18·2% of pentose (arabinose) and 11·8% of hexose (glucose). These results are in accord with the mixed nature of the gum (see Jones & Smith, 1949).

Determinations on human urine. Urine samples 1, 2, 3, 5 and 6 (Table 4) were all obtained from apparently healthy male Chinese, samples 7 and 8 from female Chinese and samples 4 and 10 from European subjects. Sample 9 was obtained from a male Chinese, aged 9 years, with subacute nephritis,

and contained 0·85 g. of protein/100 ml. Samples 2 and 3 were obtained during urine-concentration tests (e.g. Fishberg, 1939) and thus should presumably have contained dissolved material in a concentration approaching the maximum possible for normal subjects. Urine 1 was obtained after a large intake of fluid by the subject concerned. Urines 6, 7, 8 and 9 were 24 hr. specimens with total volumes of 750, 1075, 620 and 460 ml. respectively.

If a creatinine excretion of 1·7 g./day for each subject is assumed for samples 1–5, the calculated or estimated daily excretion of glucuronic acid for all the samples ranges from 0·065 to 0·67 g./day. This is just within the normal range for European subjects (Brox, 1953; Fishman *et al.* 1951; Deichmann & Thomas, 1943).

The recovery experiments for urine samples 5 and 7 illustrate the effect of insufficient dilution of the

Table 3. *Glucuronic acid and glucose contents of some acidic polysaccharides as estimated by the carbazole reaction*

All the polysaccharide preparations except heparin were dried *in vacuo* over P_2O_5 at 80° for 2 hr. before weighing. Solutions of all the preparations were in water except oxidized cellulose, which was in 0·005N-NaOH. Sulphate analyses on the solutions of chondroitinsulphate and heparin were carried out in triplicate by the method of Bray, Humphris, Thorpe, White & Wood (1952). Glucose was assumed to be nil if the estimated glucose content of the polysaccharides was not more than 2% by wt. of the estimated glucuronic acid content. Estimated pentose contents of the polysaccharides were in all cases less than 4% of the combined estimated glucuronic acid and glucose contents and were assumed to be nil in calculating the figures given for glucuronic acid and glucose.

Material	Quantity	Glucuronic acid (μmoles)	Glucuronic acid (%)	Glucose (μmoles)
Potassium hyaluronate	360 μg.	0·392	21·1	0·172
Purified hyaluronate*	155 μg.	0·289	36·3	Nil
Chondroitinsulphate (containing 2·95% N)	310 μg.†	0·529	33·1	Nil
Heparin ('Pularin' Evans; 5000 i.u./ml.)	25 i.u.‡	0·403	21·7	0·077
Oxidized cellulose	100 μg.	0·313	60·8	0·122
Bromine-oxidized starch§	140 μg.	0·113	15·6	0·522

* Aqueous potassium hyaluronate (20 mg. in 10 ml.) was shaken with 5 ml. of amyl alcohol–chloroform (1:4, v/v) for 0·5 hr. The mixture was then centrifuged at 2000 rev./min. for 0·5 hr.; about 6 ml. of the clear aqueous layer was mixed with 95% ethanol (30 ml.); this mixture was kept for 6 hr. at 4°, and the resulting precipitate of hyaluronate was separated by centrifuging.

† Contained 0·537 μmole of sulphate.

‡ 0·36 mg. dry wt. Contained 0·941 μmole of sulphate.

§ Prepared by the method of Farley & Hixon (1942).

Table 4. *Concentrations and recoveries of uronic acid and hexose in various urines, as estimated by the carbazole procedure*

Values in columns 5–8 are means and standard deviations obtained from six individual estimations of 1 ml. of diluted urine samples.

Urine	Sp.gr.	Creatinine (mg./ml.)	Dilution of urine for the carbazole reaction (ml./20 ml.)	Estimated concn. in undiluted urine (μg./ml.): Uronic acid (as glucuronolactone)	Estimated concn. in undiluted urine (μg./ml.): Hexose (as glucose)	Recoveries (μg./ml. of diluted urine) after addition of 20 μg. (50 μg. for urine 5): Glucuronolactone	Recoveries (μg./ml. of diluted urine) after addition of 20 μg. (50 μg. for urine 5): Glucose
1	1·005	0·30	2·0	62±5·6	224±10·1	18·9±0·61	19·8±0·89
2	1·033	3·40	1·0	483±14·1	607±32·7	18·5±0·96	19·2±1·29
3	1·029	4·60	1·0	315±11·3	707±28·1	18·2±0·94	20·3±1·42
4	1·010	1·34	1·0	427±9·4	433±29·4	18·5±0·35	19·4±1·27
5	1·024	2·24	0·8	755±23·8	1313±16·3	46·7±1·13	44·0±0·97
5	1·024	2·24	0·4	794±21·2	1306±31·6	48·1±1·07	49·5±0·97
6	1·016	1·85	1·0	348±18·1	561±30·3	18·5±0·31	19·1±1·28
7	1·011	0·78	1·0	55±4·1	1883±72·8	19·6±0·60	15·5±2·17
8	1·018	1·60	1·0	237±8·4	247±7·6	19·2±0·42	20·0±0·92
9	1·013	0·34	1·0	40±3·4	279±17·2	19·1±0·50	18·7±2·05
10	1·020	1·69	1·0	465±19·7	1243±63·2	17·9±1·18	20·1±2·45
10 plus acetoacetate (2 mg./ml.) and acetone (1 mg./ml.)	—	—	1·0	—	—	17·1±0·51	19·6±1·33
10 plus albumin (2 mg./ml.)	—	—	1·0	—	—	18·1±0·85	19·0±1·31
10 plus urea (50 mg./ml.)	—	—	1·0	—	—	18·3±1·31	19·6±1·92

urine on the results obtained. When 0·8 ml. of urine 5 was diluted to 20 ml. the concentration of glucuronolactone plus glucose was above the limit of 170 μg./ml. (see Methods); with urine 7 the final concentration of glucose was higher than 100 μg./ml.

The estimated pentose contents of the urine samples had little statistical significance and are not included in Table 4. In no case was the probability, that the mean of the estimates for a urine sample differed from zero by chance alone, less than 0·09.

One experiment with 40 μg. of added arabinose gave a mean recovery of $32{\cdot}85 \pm 2{\cdot}34$ μg. The estimated concentrations of glucuronolactone and glucose in this urine were 528 ± 19 and 1335 ± 42 μg./ml. respectively. The concentrations found for the urine without added arabinose were 540 ± 24 and 1300 ± 27 μg./ml.

The estimation of pentose by the standard procedure was unreliable in many urines because of variable contributions to the E_v reading by materials other than pentose. Acetone and acetoacetate, for instance, in a concentration greater than 2–3 mg./ml., gave a slight yellowish colour after 24 hr. which absorbed light in the violet region.

Guinea-pig urine. Samples of 24 hr. urine collected over 4 days from nine normal guinea pigs on a dry diet (crushed oats, bran and milk powder with appropriate vitamin and mineral supplements) showed a mean total (free plus combined) glucuronic acid excretion of $3{\cdot}34 \pm$ S.D. 1·67 mg./day/guinea pig and a glucose excretion of $7{\cdot}50 \pm 4{\cdot}70$ mg. The same guinea pigs were each injected subcutaneously with 50 mg. of borneol in olive oil on two separate days. The glucuronic acid and glucose excretions in the 24 hr. after injection were $19{\cdot}24 \pm 6{\cdot}16$ and $7{\cdot}84 \pm 6{\cdot}52$ mg. respectively.

DISCUSSION

The results in Table 2 show that the modified carbazole method can be applied to solutions containing glucuronolactone, glucose and xylose to give estimates of one or all of these constituents over the concentration range for each of 10–100 μg./ml.

Table 1 shows that the differences between the readings obtained with equimolar amounts of glucuronolactone, glucuronic acid and barium glucuronate were not significant in relation to the standard deviations given in Table 2. The readings obtained with bornyl and menthyl glucuronosides were somewhat lower than for an equimolar amount of glucuronolactone, but the magnitudes of any two of the readings were related to the third by almost the same ratios as for glucuronolactone. Table 1 also shows that fructose and soluble starch gave readings which did not differ significantly from those of an equivalent amount of glucose. Fructose gave a pink blank not given by glucose in equimolar concentration, but this colour apparently did not interfere with the development of the colour with carbazole, or the subsequent measurement against the blank. Galactose and lactose gave lower readings than an equivalent amount of glucose, but the magnitudes of any two of the readings were related to the third by almost the same ratios as for glucose. It is thus possible to use the nomogram (Fig. 1) for the estimation of glucuronic acid and its barium salt (and presumably other salts), and of fructose and the glucose in soluble starch.

An attempted purification of the heparin preparation (Table 3), unlike that of the hyaluronate preparation, failed to reduce the low estimated glucose content. The anomalous readings obtained with heparin may be characteristic of the structure of the compound rather than a result of the presence of glucose or some other hexose in the preparation. Dische (1955) states that the hexuronic acid of heparin gives anomalous results in a number of colour reactions. Dische (1947) found that the extinction coefficient of the colour obtained with heparin in the reaction with carbazole was 50% greater than that obtained with an equivalent amount of glucuronic acid. The extinction coefficient obtained for heparin in the present work does not appear to be greater than that which might be expected from the glucuronic acid content calculated from previous analyses on heparin preparations (see Foster & Huggard, 1955).

The figures obtained for gum tragacanth, together with those in Table 2, indicate the possibility that the method may be useful in testing preparations of the plant hemicelluloses, which contain glucuronic or galacturonic acid, glucose or galactose and xylose or arabinose.

The highest concentration of glucose found in nine normal urines was 0·19%. In the present method, non-carbohydrate materials and reducing glucuronosides do not contribute to the figures for glucose. The figures would, however, include, if they were present, other free or combined hexoses which occasionally occur in urine. The recovery of added glucuronolactone and glucose appeared to vary little despite the widely differing specific gravities of the urines. Albumin, urea, or acetoacetate plus acetone, when added in amounts corresponding with the average excess above normal found in pathological conditions, had little effect on the recovery from urine of either glucuronolactone or glucose. The urine of a nephritic subject also showed no decrease in the recoveries. Further recovery experiments, not shown in Table 4, indicated that addition of larger quantities of the ketone bodies decreased the recovery of glucuronolactone and that addition of larger quantities of albumin decreased the recovery of glucose, whereas the addition of larger quantities of urea gave recoveries

of glucose greater than 100%. Dische (1947) found that serum proteins in a concentration of 0·1% produced a 20% decrease in the intensity of the colour given by glucuronolactone. The failure to observe any decrease in the readings obtained with glucuronolactone in the present work may have been due to the use of a test blank, and of 0·2% (instead of 0·1%) ethanolic carbazole.

The similarity of the absorption spectra of the colour complexes of glucose and of hydroxymethylfurfural suggests that the colour from glucose is produced by reaction of carbazole with hydroxymethylfurfural formed from glucose by the action of sulphuric acid. Similarly, the colour from xylose is probably the result of a reaction between furfural and carbazole. Since the absorption spectra of the complexes from glucuronolactone at 2·5 and 24 hr. are both different from the above, and since neither furfural nor hydroxymethylfurfural gives as intense a colour at 2·5 hr. as glucuronolactone, it seems that neither of these compounds is principally responsible for the colour obtained with glucuronolactone and carbazole.

SUMMARY

1. A modification of the carbazole method for uronic acids has been adapted for the estimation of glucuronolactone, glucose and xylose in solutions containing any or all of these compounds in the concentration range 10–100 μg./ml. The results were reproducible to within ± 4 μg. except for some mixtures containing xylose.

2. The method gave good recoveries of glucuronolactone and glucose added to urine. These recoveries were not affected by the addition of albumin, ketone bodies, urea or arabinose in limited quantities.

3. The method gave figures for the concentrations of glucuronic acid in chondroitinsulphate, hyaluronate and heparin preparations, and in urine, which were of the same order as previously published figures obtained by other methods.

4. Hydroxymethylfurfural is probably responsible for the colour given by glucose, and furfural for that given by xylose. The material responsible for the colour given by glucuronic acid does not appear to be either of these compounds.

I am indebted to Mr G. M. Ching for the creatinine determinations.

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Latent Phenolase in Extracts of Broad-Bean (*Vicia faba* L.) Leaves

1. ACTIVATION BY ACID AND ALKALI

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Latent phenolase activity has been found in extracts of eggs of the grasshopper *Melanoplus differentialis* (Bodine & Allen, 1938*a*, *b*), mealworm *Tenebrio molitor* (Bodine & Allen, 1941), *Drosophila* spp. (Ohnishi, 1954; Horowitz & Fling, 1955) and crayfish serum (Bodine & Allen, 1941). The extensive investigations of Bodine and co-workers (Bodine & Boell, 1935; Bodine, Allen & Boell, 1937; Allen, Ray & Bodine, 1938; Allen & Bodine, 1941; Allen, Otis & Bodine, 1942; Bodine & Tahmisian, 1943; Bodine, Tahmisian & Hill, 1944) with extracts and preparations from grasshopper eggs have shown that active phenolase is released by treatment with certain organic solvents (e.g. acetone, chloroform),