3. Less bacteriochlorophyll and more coproporphyrinogen were produced with increasing concentrations of methionine. The production of magnesium protoporphyrin monomethyl ester was greatest at an intermediate methionine concentration.

4. The synthesis of magnesium protoporphyrin monomethyl ester by cultures or suspensions was increased 10- to 20-fold by adding Tween 80.

5. Coproporphyrinogen synthesized by *R. capsulata* was identical with the product of chemical reduction of coproporphyrin III in all properties examined. A compound with an absorption peak in acid solution at 500 m μ , probably the dipyrromethene tetrapyrrole, was intermediate in the chemical oxidation of coproporphyrinogen to coproporphyrin.

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Metabolism of D-Glucuronolactone in Mammalian Systems

3. FURTHER STUDIES OF D-GLUCURONOLACTONE DEHYDROGENASE OF RAT LIVER*

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Marsh (1963b) described the identification in certain mammalian tissues of an enzyme system capable of converting D-glucuronolactone into

D-glucaric acid, and it was suggested that this could explain the presence of D-glucaric acid in normal human urine and the greatly increased excretion of this compound immediately after oral dosage of D-glucuronolactone (Marsh, 1963a). The

* Part 2 (Marsh 1963b).

oxidative enzyme, provisionally named D-glucuronolactone dehydrogenase, was present in the soluble or 'cytoplasmic' fraction of tissue homogenates, and required 1 molecule of NAD/molecule of D-glucuronolactone oxidized. By analogy with the action of other hexose dehydrogenases, it was postulated that the primary product from D-glucuronolactone was D-glucaro-(1→4)-(6→3)-dilactone, which is spontaneously hydrolysed in aqueous solution to D-glucaric acid and its (1→4)- and (6→3)-monolactones. A further study of rat-liver D-glucuronolactone dehydrogenase is now presented.

METHODS AND MATERIALS

Enzyme preparations. The source of both D-glucuronolactone-dehydrogenase and β -glucuronidase preparations was liver tissue of adult rats (hooded Lister strain). For studies of the dehydrogenase, 'crude preparations' (non-particulate fraction from homogenates in 0.17M-KCl, containing 6.0–6.5 mg. of protein/ml.) and 'partially purified preparations' [by $(\text{NH}_4)_2\text{SO}_4$ fractionation of crude preparations, and containing 4.5–5.5 mg. of protein/ml. of final solution] were made as described by Marsh (1963b). Enzyme preparations from guinea-pig liver were made similarly.

Substrates. NAD, NADH_2 (sodium salt) and NADP (sodium salt) were 'Sigma grade' (Sigma Chemical Co., St Louis, Mo., U.S.A.), for which 97.5–100% purity was claimed. D-Glucuronolactone (Corn Products Refining Co., New York, U.S.A.), recrystallized once from water, had m.p. 175–177°. Phenolphthalein β -glucuronide was prepared biosynthetically (Levy & Marsh, 1959).

Enzyme assays. The detailed procedure for the assay of D-glucuronolactone dehydrogenase is described by Marsh (1963b); unless otherwise stated the incubation mixture (5 ml.) contained 0.05M- NaH_2PO_4 -NaOH buffer, pH 6.5, 5 mM-D-glucuronolactone, 2.5 mM-NAD and 1 ml. of the enzyme preparation. The product, after incubation for 1 hr. at 37°, was heated at pH 2.0–2.2 to convert D-glucarate partially into D-glucaro-(1→4)-lactone, then tested as an inhibitor of β -glucuronidase at pH 5.2, with 0.63 mM-phenolphthalein β -glucuronide as substrate. β -Glucuronidase assays containing no inhibitory material liberated 35–40 μg . of phenolphthalein/hr. The quantity of D-glucarate produced in the initial incubation, measured in 'inhibitor units' (u.i.), where 1 u.i. caused 50% inhibition of β -glucuronidase, was determined from a standard curve for acid-heated inhibitor. Correction was then made for the slight inhibition (usually equivalent to about 0.25 u.i.) due to the 'blank', containing buffer, D-glucuronolactone and heat-inactivated enzyme. A conversion factor (1 u.i. $\equiv$ 5.06 μg . of D-glucaric acid; Marsh, 1963a) was employed to calculate the total D-glucaric acid produced in the initial incubation.

Other procedures. Measurement of protein, NADH_2 and total uronic acid were made as described by Marsh (1963b). Optical rotation was measured on a Hilgar Microptic polarimeter with a sodium source.

Other materials. Potassium hydrogen D-glucarate ('potassium hydrogen saccharate' from British Drug Houses Ltd., Poole, Dorset) was recrystallized once from water. D-Glucaro-(6→3)-lactone, m.p. 143–145°, was

prepared as described by Smith (1944), and D-glucaro-(1→4)-lactone monohydrate (Bose, Hullar, Lewis & Smith, 1961), m.p. 90–95°, was presented by Dr H. N. Fernley. The latter compound was heated *in vacuo* over P_2O_5 for 6 hr. at 60°, then for 24 hr. at 100°, to give D-glucaro-(1→4)-(6→3)-dilactone (Smith, 1944) as a yellow glass (loss in weight on heating was 17.4%; theory requires 17.1%). D-Mannuronolactone was prepared by the hydrolysis of methyl α -D-mannosiduronic acid (Marsh, 1952). Other chemicals were obtained commercially and used without further purification.

All melting points are corrected.

EXPERIMENTAL AND RESULTS

Factors affecting the enzymic activity

In assays conducted at pH 6.5, it has been shown by Marsh (1963b) that the production of D-glucaric acid from D-glucuronolactone was proportional to the enzyme concentration over the range usually employed in a normal 1 hr. assay. Proportionality of D-glucaric acid production with the period of incubation did not extend much beyond 1 hr., however. This loss of proportionality may have been due to loss of enzyme activity, or to excessive hydrolysis of the lactone substrate, for Dowben (1959) has reported appreciable hydrolysis of D-glucuronolactone at 37° in neutral phosphate buffer. Exhaustion of the substrate by enzyme action was most unlikely to be a factor, however, for the conversion of D-glucuronolactone or of NAD did not normally exceed 5%.

Variation of enzyme activity and stability with pH. When assays were performed in 0.05M-sodium dihydrogen phosphate-sodium hydroxide buffer, a single narrow pH optimum was displayed at 6.5–6.6 (Fig. 1). Enzyme activity was less than 50% of the optimum outside the pH range 5.5–7.8.

Fig. 1. pH-activity curve for rat-liver D-glucuronolactone dehydrogenase. The partially purified preparation (1 ml.) was incubated with 5 mM-D-glucuronolactone and 2.5 mM-NAD in 0.05M-orthophosphate buffer for 1 hr. in a total volume of 5 ml.

A study of the pH stability of the crude preparation was made; samples of a single enzyme preparation were adjusted to the required pH at 0° with hydrochloric acid or sodium hydroxide, incubated for 2 hr. at 37°, cooled to 0° and the pH brought back to 6.5. Activities were then compared with an unheated sample of the enzyme (natural pH 6.8) that had been stored at 0° (Fig. 2). Maximum D-glucuronolactone-dehydrogenase activity was retained between pH 5.5 and 7.0, and preparations incubated below pH 4 or above pH 10.5 displayed no subsequent enzyme activity. Even over the range of maximum pH stability there was a significant loss (up to 10%) of activity compared with the non-incubated preparation. Preparations buffered at pH 6.5 and stored at 2° lost up to 50% of their activity within 48 hr.

Effect of buffer. When assays were performed at pH 6.5 for 1 hr. with the same crude enzyme preparation, but with different buffers (all 0.05M), there were considerable differences in D-glucarate production. Typical relative values were: sodium dihydrogen phosphate-sodium hydroxide buffer, 100; tetrasodium pyrophosphate-hydrochloric acid buffer, 105; trisodium arsenate-hydrochloric acid buffer, 86; imidazole-hydrochloric acid buffer, 53; maleic acid-sodium hydroxide buffer, 34; 3,3-dimethylglutaric acid-sodium hydroxide buffer, 26; collidine-hydrochloric acid buffer, 20. The decreased enzyme activity in assays where phosphate was not used was due to inhibitory effects of these other buffers, for when 0.05M-phosphate, pH 6.5, was also present, there was no change in the assay values. In assays with phosphate buffer alone, there was no appreciable change in D-glucaric acid production when the buffer concentration was varied (0.01–0.2M). It was essential, however, when

Fig. 2. Stability of a crude rat-liver D-glucuronolactone-dehydrogenase preparation to treatment at various pH values for 2 hr. at 37°. After treatment, the assay was made at pH 6.5 (for details see Fig. 1). The activity of the untreated preparation (100%), retained at 0° for 2 hr., was 0.40 μ mole of D-glucaric acid/ml./hr.

low buffer concentrations were used, to ensure that the pH of the incubation mixture did not fall appreciably below pH 6.5, i.e. the pH of optimum enzyme activity, owing to the influence of D-glucuronic acid produced by increased hydrolysis of the lactone by phosphate (see above).

Substrate specificity. Replacement of D-glucuronolactone in the usual enzyme assay by 5 mM-D-galacturonate, 5 mM-D-glucono-(1 $\rightarrow$ 4)-lactone or 5 mM-D-glucono-(1 $\rightarrow$ 5)-lactone produced no evidence of the formation of a β -glucuronidase inhibitor after acid treatment of the products. D-Galacturonic acid was not therefore oxidized to mucic acid (galactaric acid), for the latter, after boiling at acid pH, is a strong β -glucuronidase inhibitor (Levy, 1952). Nor was there conversion of the D-gluconolactones into D-glucaric acid derivatives (i.e. $-\text{CH}_2\cdot\text{OH} \rightarrow -\text{CO}_2\text{H}$ at C-6). The presence of 5 mM-D-mannuronolactone in the usual assay medium produced no inhibitory effect; this compound was not therefore a substrate for the enzyme, competing with D-glucuronolactone. The crude enzyme preparation (2 ml.; activity, 0.43 μ -mole of D-glucaric acid/ml./hr.) was incubated with 5 mM-2-acetamido-2-deoxy-D-glucose and 2.5 mM-NAD at pH 6.5 (total volume 5 ml.) and 37° for 1 hr. The product was boiled at pH 2.5 for 20 min., then tested as an inhibitor of rat-epididymal β -N-acetylglucosaminidase (Conchie, Findlay & Levy, 1959) at a concentration corresponding to 0.8 mM-2-acetamido-2-deoxy-D-glucose. There was no inhibition and hence no enzymic conversion into N-acetyl-D-glucosaminolactone, the specific inhibitor of β -N-acetylglucosaminidase (Findlay, Levy & Marsh, 1958), which acts at micromolar concentrations.

Substitution of 2.5 mM-NADP for NAD in the assay with D-glucuronolactone produced variable effects, but D-glucarate formation was always greatly diminished. With one sample of NADP no inhibitor was produced, and with a second sample 0.096 μ mole of D-glucaric acid was formed, compared with 0.41 μ mole when 2.5 mM-NAD was used. Decrease of the NAD concentration to 50 μ M (i.e. 50-fold) then diminished the D-glucaric acid formed in the assay to 0.14 μ mole. Thus the presence of less than 2% of NAD as an impurity in a specimen of NADP could account for an apparent positive effect by the latter. It therefore seems probable that the dehydrogenase is specific for NAD as the coenzyme.

Effect of substrate concentration. Changes in the production of D-glucaric acid with the concentrations of D-glucuronolactone and NAD were examined with partially purified preparations. It was not possible to attain enzyme saturation with D-glucuronolactone, high concentrations (e.g. > 25 mM) of which caused large 'blank' values in

the assay and led to gross errors in the determination of enzyme activity. Saturation of the enzyme by NAD was achieved at relatively low concentrations of the coenzyme, however, and inhibition by excess of substrate was apparent. The NAD concentration for maximum activity was dependent on the D-glucuronolactone concentration; thus optimum NAD concentrations were 5 mM with 20 mM-glucuronolactone, 2.5 mM with 5 mM-glucuronolactone (i.e. as employed in routine assay measurements), and 1 mM with 1.25 mM-glucuronolactone. Increasing the NAD concentrations to 10 mM, when 5 mM-glucuronolactone was used, led to 50% enzyme inhibition.

As pointed out by Dixon & Webb (1958), in a reaction involving two substrates a variation in the concentration of either substrate usually gives a curve of the Michaelis type, from which an apparent affinity constant for the variable substrate may be derived; this 'constant' is dependent on the chosen concentration of the second substrate. By employing the method of Lineweaver & Burk (1934), the apparent Michaelis constant for D-glucuronolactone was 17 mM with a fixed concentration of 2.5 mM-NAD, and the corresponding constant for NAD was 0.24 mM with a concentration of 5 mM-D-glucuronolactone (see Fig. 3). Evaluation of the true affinity constants by the graphical method of Florini & Vestling (1957) was not successful; the useful range of NAD concentrations was very narrow, upper and lower limits being imposed by substrate saturation and the accurate measurement of initial enzyme velocity respectively.

Reversal of the enzyme reaction

Experiments were conducted to investigate the possible reduction of D-glucaric acid and its lactones to D-glucuronic acid or D-glucuronolactone by a partially-purified rat-liver preparation of D-glucuronolactone dehydrogenase (activity, 0.48 μ -mole of D-glucaric acid/ml./hr.). Potassium hydrogen D-glucarate, D-glucaro-(1 $\rightarrow$ 4)-lactone or D-glucaro-(6 $\rightarrow$ 3)-lactone (25 μ moles) were incubated with NADH₂ (5 μ moles) and the enzyme preparation (1 ml.) in a total volume of 5 ml., at pH 6.5 for 1 hr. at 37°. The incubation mixtures were chilled to 0° and centrifuged, and the NADH₂ contents compared with that of a control in which the sugar acid was added at the end of the incubation period. Loss of NADH₂ (13%) occurred only in the presence of D-glucaro-(6 $\rightarrow$ 3)-lactone, and the production of uronic acid could not be detected in any of the incubation mixtures.

D-Glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone, the postulated initial product of D-glucuronolactone oxidation, was also tested, under the same conditions but with an enzyme preparation of activity 3.7 μ moles of D-glucaric acid/ml./hr., and at pH 4.2 and 5.2

(0.1 M-acetic acid-sodium hydroxide buffers) in addition to pH 6.5. Losses of NADH₂, due to decomposition when incubated alone at these pH values, were: at pH 4.2, 97%; at pH 5.2, 54%; at pH 6.5, 7%. At pH 6.5 there was a further loss of 11% of NADH₂ in the presence of the dilactone and the enzyme preparation, but in no case could uronic acid be detected at the end of the incubation period. Whereas the monolactones of D-glucaric acid are relatively stable at pH 6.5, aqueous solutions of D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone underwent a rapid decrease in optical activity even in mildly acid solution (Table 1), and after 1 hr. at pH 6.5 and 17° the specific rotation approached the equilibrium value of 31.5° quoted by Smith (1944). It therefore seemed unlikely that the dilactone could survive for any appreciable part of the enzyme incubation at 37° and pH 6.5, and at lower pH values any possible reduction was restricted by the acid-lability of NADH₂ and the decreased activity and stability of the enzyme (see Figs. 1 and 2).

Inhibition by drugs and aliphatic alcohols

The administration of a wide variety of compounds, of unrelated chemical structure, has been found to increase the excretion of L-ascorbic acid by rats, and of D-glucuronic acid by guinea pigs (Longenecker, Fricke & King, 1940; Burns, Evans & Trousof, 1957). It is now known that D-glucuronolactone, or D-glucuronic acid, is utilized in mammalian L-ascorbic acid biosynthesis, and an examination was therefore made of the effects of some drugs on D-glucuronolactone-dehydrogenase activity. Relatively low concentrations of nembutal [pentobarbitone; 5-ethyl-5-(1'-methylbutyl)-barbituric acid], pentothal [thiopental; 5-ethyl-5-(1'-methylbutyl)thiobarbituric acid], barbital (veronal; 5,5-diethylbarbituric acid), chlorotone (chlorbutol; 1,1,1-trichloro-2-methylpropan-2-ol) and chloral hydrate caused inhibition of the enzyme from rat liver, and, to a lesser extent, that from guinea-pig liver (Table 2). Of the barbiturates, nembutal and pentothal were considerably more

Table 1. *Change in optical activity of aqueous D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone solutions at different pH values*

Optical rotations were determined at 17° in 0.1M-acetic acid-NaOH buffer, pH 4.2 and 5.2, or in 0.05M-NaH₂PO₄-NaOH buffer, pH 6.5, in a 0.5 dm. micro-tube (c 1.0).

pH	Specific rotation				
	Initial	0.5 hr.	1 hr.	2 hr.	20 hr.
4.2	151°	140°	128°	—	44°
5.2	156	118	98	94°	—
6.5	106	59	41	35	—

potent inhibitors than barbitol. It was considered that the effect might be influenced by the low water-solubility and lipophilic nature of nembutal and pentothal at physiological pH. In support of this hypothesis, the inhibitory effects of an homologous series of normal primary aliphatic alcohols increased greatly with molecular weight (Table 3). Whereas methanol was almost non-inhibitory, inhibition by 5 mM-ethanol was appreciable and the inhibitory power of higher homologues rose to a limiting value attained by pentan-1-ol, after which it remained constant.

The inhibitory effects of barbitol or pentan-1-ol were not increased when either compound was pre-incubated with the enzyme for 1 hr. at 37°, in the presence or absence of NAD, before the addition of the substrate(s). They thus appear to act as true inhibitors of D-glucuronolactone dehydrogenase and not by irreversibly inactivating the enzyme.

Examination of the kinetics of inhibition (Line-weaver & Burk, 1934) by barbitol and pentan-1-ol (Figs. 3a and 3b) showed that both compounds acted non-competitively with either D-glucurono-

Table 2. *Inhibition of liver D-glucuronolactone dehydrogenase by drugs*

Crude liver preparations (see the Methods and Materials section) of rat (enzyme activity, 0.49 μ mole of D-glucaric acid/ml./hr.) and guinea pig (enzyme activity, 0.63 μ mole of D-glucaric acid/ml./hr.) were incubated with 5 mM-D-glucuronolactone and 2.5 mM-NAD at pH 6.5 for 1 hr.

Inhibitor	Inhibition of activity (%)	
	Rat enzyme	Guinea-pig enzyme
5 mM-Barbital	52	36
2 mM-Barbital	28	—
2 mM-Nembutal	65	49
2 mM-Pentothal	53	35
2 mM-Chloretone	25	16

Table 3. *Inhibition of rat-liver D-glucuronolactone dehydrogenase by normal primary aliphatic alcohols*

The enzyme activity of the crude enzyme preparation was 0.30 μ mole of D-glucaric acid/ml./hr.; alcohols were present in concentrations (a) 2% (w/v), (b) 5 mM, in the assay (for details see Table 2).

Alcohol	Inhibition of activity (%)	
	(a)	(b)
Methanol	9	2
Ethanol	64	31
Propan-1-ol	84	52
Butan-1-ol	91	70
Pentan-1-ol	98	80
Hexan-1-ol	100	75
Heptan-1-ol	100	79
Octan-1-ol	98	79

Fig. 3. Reciprocal plots for a crude enzyme preparation with: (a) variable D-glucuronolactone concentration and 2.5 mM-NAD; (b) variable NAD concentration and 5 mM-D-glucuronolactone. O, Control; Δ , with 2 mM-barbital; $\square$, with 1 mM-pentan-1-ol. Incubation with enzyme (1 ml.) was for 1 hr. at 37° and pH 6.5.

lactone or with NAD. From the illustrated experiments, values derived for K_i , the inhibitor constants, were as follows: barbital, 4.0 mM (variable D-glucuronolactone concentration), 3.0 mM (variable NAD concentration); pentan-1-ol, 0.53 mM (variable D-glucuronolactone concentration), 0.49 mM (variable NAD concentration). Since inhibition by barbital was non-competitive, the effect could not be due to the drug removing NAD during the incubation, and indeed there was negligible production of NADH_2 on incubating 5 mM-barbital with the enzyme system in the absence of D-glucuronolactone.

A variety of carcinogenic hydrocarbons, including 3-methylcholanthrene and 3,4-benzpyrene, which also markedly increase L-ascorbic acid excretion (Burns *et al.* 1960), had no effect on the enzyme system.

DISCUSSION

Rat-liver D-glucuronolactone dehydrogenase appears to have hydrogen-acceptor specificity limited to NAD, and this distinguishes the enzyme from liver D-glucose dehydrogenase which is able to utilize NAD or NADP (Strecker & Korkes, 1952). The affinity for NAD was much greater (about 100-fold) than for D-glucuronolactone, although it is emphasized that the impure nature of the enzyme preparations and the methods employed have yielded only approximate values of the Michaelis constants for either substrate. Despite the relatively low affinity for D-glucuronolactone, however, there was no evidence of action on either D-mannuronolactone or D-galacturonic acid; this supported the observation (Marsh, 1963*a*) that oral administration of D-galacturonic acid did not increase the excretion of β -glucuronidase inhibitors. It would seem that furan-2,5-dicarboxylic acid, claimed by Flaschenträger, Cagianut & Meier (1945) to be excreted in greatly increased quantities after the feeding of D-galacturonic acid to human subjects, could not have arisen from galactaric acid during the isolation procedure by a process analogous to that suggested for D-glucaric acid (Marsh, 1963*a*).

Attempts to demonstrate reversal of the dehydrogenation reaction, by employing D-glucaric acid or its lactones and NADH_2 , were unsuccessful. The demonstrated instability of D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone, which is the postulated initial oxidation product, at pH values approaching the optimum activity of the dehydrogenase and at which NADH_2 is stable in solution, would seem to provide a reasonable explanation for this, irrespective of the equilibrium position of the catalysed reaction. It is also unlikely that any of the other D-glucaric acid derivatives tested could, even if appropriate lactonases were present, be converted into the dilactone at pH 6.5. On these assumptions,

the oxidative reaction is in effect irreversible. Slight losses of NADH_2 in the presence of D-glucaro-(6 $\rightarrow$ 3)-lactone or the dilactone could not be ascribed to D-glucuronolactone-dehydrogenase action, since there was no concomitant production of uronic acid.

Inhibition of the enzyme by drugs and aliphatic alcohols would appear to be a non-specific effect, but is of particular interest in that $\text{NADP-L-hexonate dehydrogenase}$, which reduces D-glucuronolactone to L-gulonolactone and is believed to be an essential enzyme in the L-ascorbic acid biosynthetic pathway, was also found to be powerfully but non-competitively inhibited by barbital (Mano, Suzuki, Yamada & Shimazono, 1961). The inhibitory effect on D-glucuronolactone dehydrogenase might offer an explanation for the well-established drug-induced biosynthesis of L-ascorbic acid, in that a block of the D-glucaric acid 'shunt' would provide larger quantities of D-glucuronic acid or its lactone for conversion into the vitamin. Preliminary observations have shown, however, that the administration of barbital to rats increases the excretion of D-glucaric acid even more spectacularly than that of L-ascorbic acid. This would suggest that barbiturates increase the production *in vivo* of free D-glucuronic acid, a conclusion previously reached by other workers (see Conney, Bray, Evans & Burns, 1961).

SUMMARY

1. Rat-liver D-glucuronolactone dehydrogenase had a pH optimum at about pH 6.5 and maximum stability over the range pH 5.5–7.0.

2. The enzyme was specific for NAD and did not oxidize D-galacturonic acid or D-mannuronolactone. The affinity of the enzyme for NAD was considerably greater than that for D-glucuronolactone.

3. No reversal of the reaction could be demonstrated with D-glucaric acid or its lactones and NADH_2 as substrates.

4. Non-competitive inhibition of the enzyme, obtained from rat or guinea-pig liver, was exhibited in the presence of barbiturates and of aliphatic alcohols. The magnitude of this effect increased with the water-insolubility of the inhibitors.

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The Preparation of ^{131}I -Labelled Human Growth Hormone of High Specific Radioactivity

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The radio-immuno-electrophoretic technique for the assay of insulin in human sera developed by Yalow & Berson (1960) has been applied to glucagon (Unger *et al.* 1959) and to growth hormone (Hunter & Greenwood, 1962*b*). The ability of ^{131}I -labelled hormones to compete with unlabelled hormones for antibody to the unlabelled hormone has also been utilized in assays based on the precipitation of antigen-antibody complex for glucagon (Grodsky, Hayashida, Peng & Geschwind, 1961) and for human growth hormone (Utiger, Parker & Daughaday, 1962). Immuno-electrophoretic procedures require the routine preparation of small amounts of ^{131}I -labelled hormones of very high specific radioactivity. The method used successfully for insulin by Yalow & Berson (1960) requires the handling of 30–80 mc of ^{131}I iodide in volatile solvents. Samols & Williams (1961) have used 10–20 mc of ^{131}I iodine monochloride (McFarlane, 1958) for the labelling of insulin but no experimental details are available. Human growth hormone has been iodinated by Utiger *et al.* (1962) by using modifications of the method of Yalow & Berson (1960) and Staub, Springs & Elrick (1957). They report specific radioactivities of up to $5\mu\text{C}/\mu\text{g.}$, with 0.25–2.0 atoms of total iodine/molecule, by using 10–20 mc of carrier-free sodium ^{131}I iodide and 0.2–1.0 mg. of human growth hormone. This level of specific radioactivity,

though adequate for methods based on precipitation, is inadequate for the immuno-electrophoretic technique.

The following method has been developed to allow the rapid preparation of 1.5–2.5 $\mu\text{g.}$ of ^{131}I -labelled human growth hormone with specific radioactivities of $250\text{--}590\mu\text{C}/\mu\text{g.}$ (60–75% efficiency) by using 2–4 mc of carrier-free sodium ^{131}I iodide and 5 $\mu\text{g.}$ of human growth hormone. Chloramine-T is used as the oxidizing agent. The ^{131}I -labelled hormone is calculated to contain up to 1.2 atoms of iodine/molecule.

In these preparations there are no discrete immunologically inactive components. With specific radioactivities of up to $300\mu\text{C}/\mu\text{g.}$ the preparations are quantitatively identical with the unlabelled hormone as measured by an immuno-electrophoretic method (Hunter & Greenwood, 1962*b*). At 500 and $750\mu\text{C}/\mu\text{g.}$ there is a progressive loss of affinity for the rabbit anti-(human growth hormone) serum.

A preliminary report of the method has been published (Hunter & Greenwood, 1962*a*).

MATERIALS

Buffers. All buffers were sodium salts.

Human growth hormone. Human growth hormone (Raben, 1959) is referred to as growth hormone. Solutions for iodination were made by adding growth hormone (5 mg.)