Biochemical Pharmacology, 1963, Vol. 12, pp. 105–112. Pergamon Press Ltd., Printed in Great Britain.

STUDIES ON GLUCOSACCHAROLACTONES—I GLUCOSACCHARO-1:4-LACTONE*

AKIO KIYOMOTO,† SHOICHI HARIGAYA, SATOSHI OHSHIMA and TAKASHI MORITA

Department of Biochemistry, Tokyo Research Laboratory, Tanabe Seiyaku Co., Ltd., Todamachi, Saitama, Japan

(*Received* 15 *March* 1962; *accepted* 17 *October* 1962)

Abstract—Glucosaccharo-1:4-lactone, the specific and potent inhibitor of β-glucuronidase, was orally administered to mice, rats, and guinea pigs. The results indicate that glucosaccharo-1:4-lactone is absorbed from the intestinal tract and distributed throughout the body. The glucuronidase activity of liver and kidney was inhibited for at least 2 hr after administration of the lactone and recovered gradually to the initial activity within several hours. Ten to twenty per cent of the lactone orally administered to rats was recovered in the urine afterward. These experiments indicate that there is no enzymatic ring opening of glucosaccharo-1:4-lactone in rat liver and kidney. Administration of glucosaccharate, glucuronate, glucuronolactone, and gluconolactone showed little or no effect on the tissue glucuronidase activities.

GLUCURONIDE formation is one of the effective detoxication mechanisms in animals. Administration of glucuronic acid to potentiate this detoxication system seems to be ineffective, since the free glucuronic acid does not participate in the detoxication process,[1] and the conjugation occurs through the transglucuronidation from uridine diphosphoglucuronic acid,[2–5] produced by oxidation of uridine diphosphoglucose.[6] However, because the glucuronide once formed may partly be hydrolyzed by the action of β-glucuronidase, the inhibition of this enzyme may cause an apparent increase in the amount of conjugated glucuronide. It is of interest, therefore, to determine the results of administering glucosaccharo-1:4-lactone (SL-1:4) orally to experimental animals.

The present paper is specifically concerned with (1) the absorption of orally administered SL-1:4 from the gastrointestinal tract, (2) its inhibition of liver and kidney glucuronidase activity, (3) its urinary excretion, and (4) the possibility of enzymatic ring opening of SL-1:4 *in vivo*. In addition, the spontaneous formation of glucosaccharate from SL-1:4 was investigated *in vitro* at various pH's, because glucosaccharate has a very weak inhibitory action on β-glucuronidase.

In these experiments, the calcium salt of SL-1:4, which was prepared for pharmaceutical purposes, was also used.

MATERIALS

Glucosaccharo-1:4-lactone and 3:6-lactone (*SL-3:6*) were prepared from potassium hydrogen saccharate according to Smith.[7] (SL-1:4) mp 90 to 93°, $[\alpha]_D^{26} = +37{\cdot}0°$, Found: C, 34·47%, H, 4·90%; calculated for $C_6H_8O_7 \cdot H_2O$: C, 34·29%, H, 4·76%, (SL-3:6), mp 140 to 145°, $[\alpha]_D^{22} = +48{\cdot}9°$, Found: C, 37·90%, H, 4·17%; calculated

* A preliminary report of this work appeared in *Nature, Lond.* **191**, 1298 (1961).

† Present address: Dept. of Biochemistry, School of Medicine, University of Utah, U.S.A.

for $C_6H_8O_7$: C, 37·5%, H, 4·2%.) Infrared absorption spectra showed the presence of a γ-lactone ring (1,770 cm^{-1}) and free carboxyl radical (1,725 cm^{-1}). The paper chromatography was carried out using the upper layer of a mixture of BuOH:EtOH: H_2O (4:1:5) as solvent. Both SL-1:4 (Rf, 0·45) and SL-3:6 (Rf, 0·33) showed a single spot when sprayed with an alkaline hydroxylamine solution followed by an acid ferric chloride solution.[8]

Calcium salt of SL-1:4 (SL-1:4-Ca) was prepared by mixing a methanol solution of SL-1:4 with an aqueous solution of calcium acetate, followed by addition of excess methanol or isopropanol which completely precipitated SL-1:4 as the Ca salt. The precipitate was washed with methanol and dried over calcium chloride in a desiccator. When paper chromatography was carried out with an aqueous solution of the salt, the spot remained on the starting line, but when the salt was dissolved in 0·5 N HCl in amounts equimolar to the Ca ions, it showed a single spot with an Rf value the same as SL-1:4. (Found: C, 29·11%, H, 4·27%, Ca, 8·08%; calculated for $C_{12}H_{14}O_{14}Ca \cdot 4H_2O$: C, 29·15%, H, 4·45%, Ca, 8·10%.)

Glucono-γ-lactone was synthesized from Ca gluconate by the method of Pasternack and Cragwall.[9]

Glucuronolactone was obtained from a commercial source.

Phenolphthalein glucuronide (PPG) was prepared by a modification of Fishmans' method.[10] Cinchonidine salt of PPG (157·6 mg; 2×10^{-4} mole) was suspended in 10 ml of water and made alkaline with 1 ml of 1·0 N NaOH. The free cinchonidine liberated was filtered off and washed several times with a small volume of water. The filtrate and washings were neutralized with 1·0 N HCl, diluted with water to 20 ml, and stored in a refrigerator. An aliquot of the solution was diluted tenfold with 1·0 N HCl and hydrolyzed in a sealed tube by heating in an autoclave for 30 min at 20 pounds. The concentration of phenolphthalein (PP) liberated from PPG was determined colorimetrically according to Fishman.[10] By this method, 97 to 99% of PPG(PP), present originally with the cinchonidine, was recovered in the stock solution. In the following experiments, this stock solution was diluted to 5×10^{-3} M.

o-Aminophenol glucuronide (o-APG) was prepared by the procedure of Williams.[11]

β-Glucuronidase was prepared from rat liver according to Levvy,[12] with a slight modification. Fresh liver was homogenized in 9 volumes of water. After adding 1 volume of 1·0 M acetate buffer, pH 5·2, the homogenate was incubated at 37° overnight and centrifuged at 1,500 × *g* for 5 min. To the supernatant was added an equal volume of saturated $(NH_4)_2SO_4$ solution, and the precipitated enzyme fraction was separated by centrifugation at 1,500 × *g* for 15 min, taken up in water (10 ml for 1 g of wet liver), and stored in a refrigerator. Activity of this enzyme was about 4,000 Fishman's phenolphthalein units/ml. For the present enzymatic assay, this β-glucuronidase solution was diluted with water to tenfold volume.

Experimental animals: male CF 1 mice and ddN mice, both supplied by CLEA (Central Laboratories for Experimental Animals, Tokyo), and male Wistar-Makino rats and hybrid guinea pigs were used for the experiments.

METHODS AND RESULTS

Formation of free saccharate from SL-1:4

Aqueous solutions of crystalline SL-1:4, containing 500 μg/ml and buffered at various pH's, were kept at room temperature. At various time intervals an aliquot of

each solution was taken to determine the remaining lactone by the hydroxylamine-ferric chloride method of Hestrin.[13]

As illustrated in Table 1, equilibrium was attained generally in 7 to 13 days. In the physiological pH range, the lactone disappeared faster than at lower pH's; however, over 30% of the lactone remained after the first 5 hr.

TABLE 1. THE AMOUNTS OF LACTONES IN AQUEOUS SOLUTION OF SL-1:4 (500 μg/ml) LEFT FOR VARIOUS TIMES AT VARIOUS pH'S AT ROOM TEMPERATURE*

Time after preparation of the mixture	Amount of lactones (%)							
	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 7·6	Aq. dest.
0 hr	100	100	100	100	100	100	100	100
1 hr	100	100	100	92	90	83	75	100
2 hr	96	96	94	87	87	75	56	94
5 hr	96	95	91	85	84	67	39	83
1 day	87	87	79	75	67	29	6	79
2 days	76	77	66	62	52	14	3	67
3 days	68	69	58	52	44	7	0	65
4 days	64	64	52	46	31	2	0	59
7 days	51	50	33	31	23	0	0	46
13 days	50	47	34	23	14	0	0	37

* SL-1:4 was dissolved (500 μg/ml) in 0·1 M phosphate or acetate buffer and kept at room temperature. Aliquots of the solutions were taken at various times to measure the amount of remaining lactone by the hydroxylamine method.

This study does not indicate the presence of lactonase which affects SL-1:4, since the decomposition of the lactone in the presence of liver enzyme at pH 7 and 37° was only slightly faster than by spontaneous reaction with the heated enzyme. In this respect, SL-3:6 behaved similarly to SL-1:4, whereas glucuronolactone was hydrolyzed significantly faster by liver enzyme. The results are shown in Table 2.

There is some possibility that lactones other than SL-1:4, which are sensitive to hydroxamate reaction, may be produced in the SL-1:4 incubation mixture, especially after equilibrium is attained. This was tested in the following way. Solutions of potassium hydrogen saccharate, which contained no lactone, and solutions of pure crystalline SL-1:4 were adjusted respectively to various pH's and left standing for 14 days at room temperature. As shown in Fig. 1, the total amount of lactones present after 14 days was equal in solutions of the same pH, irrespective of the two starting materials. Furthermore, paper chromatograms of these solutions demonstrated the presence of SL-3:6 as well as SL-1:4. This demonstrates that free saccharate once produced can form other lactones.

Urinary excretion of SL-1:4

The urinary excretion of SL-1:4 was measured by its inhibitory action on a known enzyme-substrate mixture of β-glucuronidase and PPG. In the case of competitive inhibition, the degree of inhibition, h, is a function of the concentration of inhibitor, I[14]:

$$h = I/(I + \phi)$$

where ϕ is $K_I(S + K_S)/K_S$ and ϕ becomes constant when the substrate concentration, S, and the dissociation constants of substrate-enzyme complex and inhibitor-enzyme complex, K_S and K_I respectively, are fixed. Therefore, when the experiments are carried out at optimal pH and at constant substrate concentration, the plot of h

against log I, upon addition of various amounts of inhibitor, gives a typical sigmoid curve in the coordinate system. Within the range of 30 to 70% inhibition, h is proportional to log I.

In order to obtain a standard curve for the inhibitory effect of SL-1:4, the following experiment was carried out. Known amounts of SL-1:4 and a standard enzyme-substrate solution containing 0·5 ml of 5×10^{-3} M PPG, 0·5 ml of β-glucuronidase solution, and 1 ml of 0·5 M acetate buffer, pH 5·2, were incubated for 1 hr at 37°, and the liberated PP was measured colorimetrically according to Fishman.[10] The resulting

TABLE 2. RING OPENING OF SACCHAROLACTONES AND GLUCURONOLACTONE IN THE PRESENCE OF LIVER HOMOGENATE

		Decrease (%) of lactone in 1·5 hr		
		SL-1:4	SL-3:6	Glucuronolactone
pH 7·0	Spontaneous	18	8	21
	With liver susp.	21	14	71
	(Difference)	(3)	(6)	(50)
pH 6·1	Spontaneous	14	5	21
	With liver susp.	14	5	28
	(Difference)	(0)	(0)	(7)
pH 4·6	Spontaneous	11	5	1
	With liver susp.	11	5	1
	(Difference)	(0)	(0)	(0)

One-half ml of a rat liver homogenate (1:1, prepared with isotonic KCl solution) was incubated with 0·5 ml of substrate solution (1·5 mmole/ml) and 4·0 ml of 0·154 M phosphate buffer for 1·5 hr at 37° for measurement of lactone ring opening. Remaining lactone was estimated by the hydroxylamine method. Spontaneous ring opening was measured similarly, using boiled homogenate.

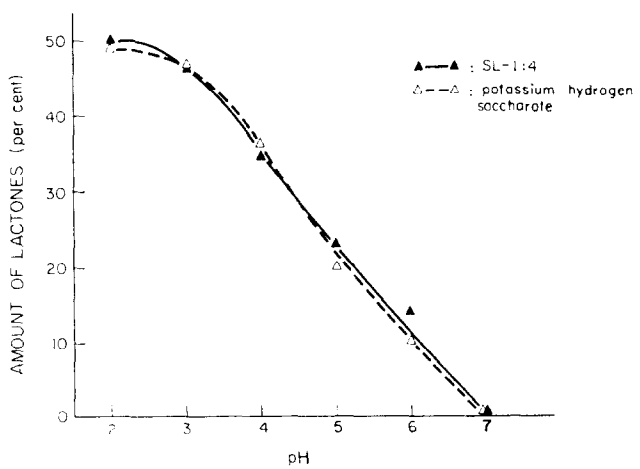

FIG. 1. Ratio of the concentration of total lactones at equilibrium to the initial concentration of SL-1:4 and potassium hydrogen saccharate. SL-1:4 (▲——▲) and potassium hydrogen saccharate (△— — —△) were dissolved (500 μg/ml) in buffer solutions of various pH's. After 14 days the amounts of total lactones were measured by hydroxylamine method. The figure illustrates the relationship between the pH of the solution and the amount of lactones at equilibrium. The amounts of lactones are shown in per cent of the initial amount of SL-1:4 and potassium hydrogen saccharate, respectively.

values were plotted as explained above, and this curve was used as a standard to measure the SL-1:4 concentration in the urine (see Fig. 2). The urine collected after SL-1:4 administration was diluted to various volumes (20 to 320 times dilution) and an aliquot was added to the standard mixture. The diluted urines showing 30 to 70% inhibition were used to calculate the amount of SL-1:4 excreted during these experiments.

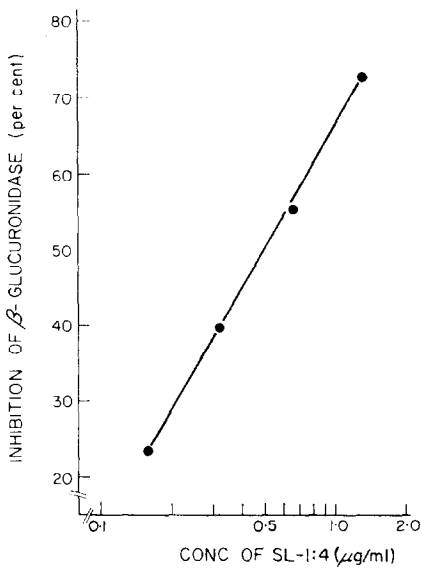

FIG. 2. Standard curve for enzymatic assay of SL-1:4. Standard substrate-enzyme mixture consisted of 0·5 ml of 5×10^{-3} M phenolphthalein glucuronide solution, 1 ml of 0·5 M acetate buffer, pH 5·2, and 0·5 ml of β-glucuronidase. The solutions of SL-1:4 added were 0·16, 0·32, 0·65, and 1·30 μg/ml. Two ml of the solution or water was added to the standard mixture and incubated at 37°. After 1 hr 2 ml of the incubated solution was deproteinized with 2 ml of 0·5% trichloroacetic acid, and the amount of released phenolphthalein was measured by Fishman's method. The standard curve was drawn through the average values of 5 experiments for each concentration of SL-1:4.

In 24 hr after oral administration of 10 mg of SL-1:4, 1·0 to 2·5 mg of unchanged lactone was found in the urine by this assay method (Table 3). However, the total amount of SL-1:4 actually excreted in the urine is unknown, owing to partial spontaneous hydrolysis during urine collection.

In these experiments, inhibitors in the urine other than SL-1:4 were measured in rats not given the lactone. The degree of inhibition shown by these urine samples was only 0 to 3%; hence any correction due to the presence of endogenous inhibitor was not necessary in these experiments.

Inhibition of liver and kidney β-glucuronidase by oral administration of SL-1:4

Freshly prepared solutions of SL-1:4 and other compounds were administered orally to the experimental animals by stomach tube. After various time intervals, the animals were sacrificed by bleeding and the organs were rapidly removed, washed with cold isotonic KCl solution, and homogenized under chilling with twofold volumes of the KCl solution in a Potter-type glass homogenizer.

One-half milliliter of the homogenate was added to a mixture containing 1 ml of 0·5 M acetate buffer (pH 5·2), 0·5 ml of substrate solution, and 2 ml of water. In the experiments with PPG as substrate, its final concentration in the incubation mixture was $6{\cdot}25 \times 10^{-4}$ M. After 1-hr incubation at 37°, the free phenolphthalein enzymatically released was spectrophotometrically measured by the procedure of Fishman.[10] In the case of *o*-APG hydrolysis, the final concentration of the substrate was $1{\cdot}25 \times 10^{-4}$ M, and after incubation for 3 hr at 37°, the remaining amount of *o*-APG was measured by the method of Levvy.[15]

TABLE 3. URINARY EXCRETION OF SL-1:4 IN 24 HR AFTER ORAL ADMINISTRATION

Animal no.	SL-1:4 (μg) excreted in urine			
	0–3 hr	3–6 hr	6–24 hr	0–24 hr
1	190	290	670	1,150
2	250	240	830	1,350
3	290	300	1,900	2,490
4	590	300	380	1,270
5	110	860	960	1,930
Average	286	398	954	1,638 (939–2,337)*

* Confidence limit, 95%.

SL-1:4 was administered 10 mg/animal orally to rat and the urine collected was diluted 20, 40, and 80 times with water. Two ml of the diluted urine samples was added to 2 ml of the standard substrate-enzyme mixture, described in Fig. 2, and after 1-hr incubation at 37° the degree of inhibition was measured. The concentration of SL-1:4 in the urine is obtained by the standard curve of Fig. 2. See text.

TABLE 4. ACTIVITY OF MOUSE LIVER β-GLUCURONIDASE 30 MIN AFTER ORAL ADMINISTRATION OF GLUCOSACCHARO-1:4-LACTONE AND ITS Ca SALT

Dosage (mg/kg)	Amounts of phenolphthalein released (μg/g tissue; incubated for 1 hr at 37°)*		Per cent of activity	
	SL-1:4	SL-1:4-Ca	SL-1:4	SL-1:4-Ca
0	1,143 ± 108	1,177 ± 148	100	100
50	949 ± 64	779 ± 71	83	76
100	824 ± 160	740 ± 156	72	63
200	550 ± 113	610 ± 195	48	52
400	466 ± 83	414 ± 136	41	35
800	279 ± 53	460 ± 260	24	39

* Mean value of five mice ($P = 0{\cdot}05$).

The experiments were carried out at first with CF 1 mice given various doses of SL-1:4 by stomach tube. The activity of liver β-glucuronidase measured, using PPG as substrate (Table 4), was slightly inhibited after oral administration of 50 mg of SL-1:4 kg; 50% inhibition was caused by 200 mg/kg. The kidney was similarly affected.

The change of liver β-glucuronidase activity with time was as follows: after administration of 400 mg of SL-1:4/kg, the enzyme action was found most strongly depressed

in 30 min and 60 min as shown in Table 5, and recovered gradually thereafter almost to the normal value. Calcium salt of SL-1:4 exhibited an effect similar to SL-1:4.

Administration of saccharate and other lactones, such as glucuronolactone and gluconolactone, had little or no effect on tissue β-glucuronidase. The results are shown in Table 6.

TABLE 5. ACTIVITY OF MOUSE LIVER β-GLUCURONIDASE AT VARIOUS TIMES AFTER ORAL ADMINISTRATION OF GLUCOSACCHARO-1:4-LACTONE AND ITS Ca SALT (400 MG/KG)

Time after administration	Amounts of phenolphthalein released (μg/g tissue; incubated for 1 hr at 37°)*		Per cent of activity	
	SL-1:4	SL-1:4-Ca	SL-1:4	SL-1:4-Ca
0	1,231 ± 161	1,208 ± 106	100	100
30 min	503 ± 101	643 ± 154	41	53
1 hr	460 ± 40	603 ± 102	37	50
2 hr	1,005 ± 173	899 ± 413	82	74
4 hr	1,169 ± 33	1,056 ± 253	95	87
6 hr	1,077 ± 131	1,093 ± 279	87	90

* Mean value of 5 mice ($P = 0.05$).

TABLE 6. ACTIVITY OF MOUSE LIVER β-GLUCURONIDASE 30 MIN AFTER ORAL ADMINISTRATION OF SOME SUGAR LACTONES

Compounds	Per cent of activity*
Control	100
Glucuronolactone, 1,000 mg/kg	90
Gluconolactone, 1,000 mg/kg	95
Potassium hydrogen saccharate, 1,000 mg/kg	86
SL-1:4, 800 mg/kg	24

* Mean percentage of the result with 5 mice.

Similar changes of liver and kidney β-glucuronidase activities were observed in experiments with ddN mice, Wistar-Makino rats, and hybrid guinea pigs. Estimation of β-glucuronidase, using *o*-APG as a substrate in place of PPG, gave similar results.

DISCUSSION

The metabolism of saccharate and SL-1:4 is not clear at present. Karunairatnum and Levvy[16] observed that the growth of mice was not affected by long-term administration of potassium hydrogen saccharate and suggested that this sugar derivative is not toxic and that it is rapidly excreted, while a small part of it may be metabolized. Bear and Blum[17] reported that glycosuria, acidosis, and increased urinary nitrogen excretion by a phlorhizinized dog could be mitigated by glucosaccharate injection. In respect to the origin of glucosaccharate, its probable formation from gluconic acid by oxidation was doubted in early studies by Bear and Blum[17] and by Schott[18]; Stetten and Stetten[19] showed later, however, that gluconic acid may be oxidized in rat to saccharic acid, but only to a small and negligible extent.

Marsh[20] reported recently that the administration of glucurono-6:3-lactone to rats increases the excretion in the urine of a potent inhibitor which they assumed to be SL-1:4. An NAD-dependent enzyme was found in the liver which is responsible for the formation of this inhibitor from glucurono-6:3-lactone.

SL-1:4 undergoes spontaneous ring opening, but the reaction is not significantly accelerated by liver enzyme. This is in contrast to the findings for glucuronolactone. It is expected from the results of experiments *in vitro* that within 2 or 3 hr after oral administration more than half of the lactone would remain unchanged. The animal experiments suggest that 10 to 20% of SL-1:4 taken orally is excreted in the urine. Because orally administered SL-1:4 could be found in liver and also in kidney, in amounts sufficient to exhibit a depression of β-glucuronidase, it would seem that the lactone is absorbed from the intestinal canal and distributed to the various tissues of the animal.

If it is assumed that any glucuronide once produced can undergo hydrolytic cleavage by β-glucuronidase, then the inhibition of this enzyme would enhance the detoxication process by increasing the concentration of glucuronides and their excretion. In this respect it is very interesting that Boyland *et al.*[21] recommended the oral administration of SL-1:4 for men exposed to carcinogenic aromatic amines and for patients who have had bladder cancers removed or destroyed. According to them, this treatment would reduce the amount of free carcinogenic aromatic amines in urine which would be released enzymatically from their glucuronides by the action of urinary β-glucuronidase. The recent report of Hartiala and Häkkines[22] that gastric ulcer formation in chicken, experimentally produced by cinchophen administration, could be inhibited by treatment with SL-1:4 solution, prepared by boiling saccharate solution, is another interesting aspect of the pharmacology of SL-1:4.

Acknowledgement—We wish to express our thanks to Professor Emeritus S. Akamatsu for his advice and interest in this work.

REFERENCES

1. J. F. DOUGLAS and C. G. KING, *J. biol. Chem.* **203**, 889 (1953).
2. G. J. DUTTON and I. D. E. STOREY, *Biochem. J.* **57**, 275 (1954).
3. K. J. ISSELBACHER and J. AXELROD, *J. Amer. chem. Soc.* **77**, 1070 (1955).
4. G. J. DUTTON, *Biochem. J.* **64**, 693 (1956).
5. J. AXELROD and J. K. INSCOE, *Nature, Lond.* **179**, 538 (1957).
6. G. J. DUTTON, *Biochem. Pharmacol.* **6**, 65 (1961).
7. F. SMITH, *J. chem. Soc.* 634 (1944).
8. F. FEIGL, V. ANGER and O. FREHDEN, *Mikrochemie* **15**, 9 (1934).
9. R. PASTERNACK and G. O. CRAGWALL, U.S. Patent no. 1,830,618.
10. W. H. FISHMAN, B. SPRINGER and R. BRUNETTI, *J. biol. Chem.* **173**, 449 (1948).
11. R. T. WILLIAMS, *Biochem. J.* **37**, 329 (1943).
12. G. A. LEVVY, *Biochem. J.* **52**, 464 (1952).
13. S. HESTRIN, *J. biol. Chem.* **180**, 249 (1949).
14. T. YANAGIDA, Advance of Enzymology (in Japan) vol. I, p. 285. Kyoritsu Press, Tokyo (1949).
15. G. A. LEVVY and I. D. E. STOREY, *Biochem. J.* **44**, 295 (1949).
16. M. C. KARUNAIRATNUM and G. A. LEVVY, *Biochem. J.* **44**, 599 (1949).
17. J. BEAR and L. BLUM, *Arch. exp. Path. Pharmacodyn.* **65**, 1 (1911).
18. E. SCHOTT, *Arch. exp. Path. Pharmacodyn.* **65**, 35 (1911).
19. M. R. STETTEN and O. STETTEN, JR., *J. biol. Chem.* **187**, 241 (1950).
20. C. A. MARSH, *Biochem. J.* **79**, 21P (1961).
21. E. BOYLAND, D. M. WALLACE and D. C. WILLIAMS, *Brit. J. Cancer* **11**, 578 (1957).
22. K. HARTIALA and I. HÄKKINES, *Acta physiol. scand.* **49**, 92 (1960).