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THE RELATIONSHIP BETWEEN 3 α-HYDROXYSTEROID NICOTINAMIDE NUCLEOTIDE COENZYME TRANSHYDROGENATION AND β-GLUCURONIDASE IN REGENERATING RAT LIVER

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Abstract—The relationship between β-glucuronidase levels and the 3α-hydroxysteroid dependent nucleotide coenzyme transhydrogenase has been investigated in regenerating rat liver tissue at various times after partial hepatectomy. It was shown that there is a direct relationship between the β-glucuronidase levels in liver and the transhydrogenase activity which is reflected as a decrease in demonstrable transhydrogenation on the addition of appropriate steroid. It was concluded that the β-glucuronidase functions so as to release the 3α-hydroxysteroid from its conjugate *in vivo*.

The effect of treating animals with the antimetabolites, 6-mercaptopurine or methotrexate on the rate of regeneration of liver was investigated as was treatment with glucosaccharo-1→4-lactone. Each of these substances causes a significant decrease in growth rate of regenerating liver and a corresponding slowing of the rate of increase of the enzymes.

In-vitro experiments showed that the 3α-hydroxysteroid transhydrogenase is not stimulated by the 3αconjugate but can be activated by the free steroid released from the glucuronic acid conjugate by β-glucuronidase. The significance of these findings in fast growing tissues is discussed.

IT WAS shown by Tomkins[1] that rat liver contained a 3α-hydroxysteroid dehydrogenase which was capable of transferring hydrogen from the steroid to either NAD or NADP. Hurlock and Talalay[2] showed that the enzyme which they obtained from rat liver caused the transfer of hydrogen between NADP and NAD and was activated by androsterone or aetiocholanolone and these workers believed that the transhydrogenase activity was the sum of NAD and NADP mediated dehydrogenases bringing this system into line with their views on the oestradiol-17β activated system from human placenta. This latter system has been much more widely investigated than the androsterone-3α system. The physiological importance of this system is still open to question but a similar system has been shown to exist in human breast and liver and in rat breast and prostate (Williams, *et al.*[3]; Baron *et al.*[4]). In the course of some experiments on the stability of the enzyme, Baron *et al.*[5] noticed that the apparent loss of activity of the enzyme on standing was due to an increase in the background rather than a decrease of the androgen activity and they suggested that this effect was due to the breakdown of a steroid conjugate present in the crude enzyme preparation, possibly as a glucuronide. Baron *et al.*[6] further investigated the purification and properties of this same enzyme.

The present work deals with the relationship between the androsterone dependent transhydrogenase activity and the β-glucuronidase activity of the regenerating tissue. This work forms a basis for future experiments on the transplantation of steroid dependent tumours into liver and the effect of antimetabolites on these tumours. The β-glucuronidase activity of regenerating rat liver has been investigated previously by several workers including Mills *et al.*[7] and Adams.[8]

MATERIALS AND METHODS

Liver enzyme preparations

Female Sprague Dawley rats were anaethetised with ether and their livers perfused *in vivo* with 0·9% sodium chloride solution containing heparin (approximately 5 units/ml). The perfusion was carried out by inserting the needle of an intravenous infusion apparatus into the hepatic artery. After treating for 2 min the hepatic vein was cut and the perfusion continued until most of the blood had been removed from the liver. The livers from 3 rats were then removed, cooled in ice, cut into small pieces and suspended in an ice-cold solution of 0·01 M nicotinamide, 0·001 M cysteine and 0·001 M EDTA in glass distilled water containing 20% glycerol (ANALAR grade), (1 ml of homogenising medium to 1 g of rat liver). The suspension was treated by an ultra-Turrax homogeniser for ten seconds with cooling. The homogenate was ultracentrifuged (105,000 *g* for 1 hr) and the clear supernatant solution removed and maintained at 0°. This has been referred to as the crude extract throughout.

The extract was purified by drop-wise addition of ice-cold saturated ammonium sulphate and the fraction precipitated between 50 and 75% saturation was collected by centrifugation (37,000 *g* for 30 min). The ammonium sulphate (ANALAR grade) had previously been purified by recrystallisation from hot water, a saturated ammonium sulphate solution prepared and neutralised with ammonium hydroxide. The precipitated fraction was dissolved in a minimum of distilled water, transferred to a dialysis bag made from 'Visking' tubing and dialysed for 15–18 hr against two changes of distilled water (2 l.) containing 0·0005 M tris-buffer (pH = 7·4) and EDTA (100 mg) at 0°. The suspension obtained from the dialysis bag was centrifuged (50,000 *g* for 10 min) and the clear supernatant decanted.

A sample of the dialysed protein solution was applied to a DEAE-cellulose column and the column eluted with 30–40 ml of 0·1 M tris-buffer pH 7·5. The eluate was collected in 2-ml portions and tested for protein and transhydrogenase activity. The column eluates containing the transhydrogenase activity were pooled and the protein was reprecipitated at 75% ammonium sulphate-saturation. The precipitated protein was separated by centrifuging and dialysed as described above, (cf. Baron *et al.*[6]). The purified enzyme was heat treated as described by Jarabak *et al.*[9] and stored in separate 1-ml portions at −20°. Each portion was heated rapidly to 0° immediately before use as it was observed that loss of activity follows repeated thawing and freezing.

Transhydrogenase assay

The assays were carried out on a spectrophotometer with automatic recording, at 20°. All four tubes contained:

Tris/HCl buffer 0·1 M pH = 7·5	0·10 ml

Glucose-6-phosphate dehydrogenase diluted to 1 mg of protein in 1 ml with 3·3 M ammonium sulphate solution pH 7 (Baron *et al.*[6])	0·01 ml
Glucose-6-phosphate di-sodium salt 0·06 M	0·05 ml
NADP mono-sodium salt 0·001 M	0·10 ml

To tubes 1 and 2, a solution of acetone–water (0·01 ml, 1:1) was added and to tubes 3 and 4 a solution of androsterone in acetone–water (1:1) (0·01 ml containing 1 mg of steroid). A further 0·1 ml of tris-HCl buffer (0·1 M pH = 7·5) was added to tube 1, while 0·1 ml of a solution of the enzyme was added to each tube and at Zero time NAD (0·1 ml 0·0075 M) was added to tubes 2, 3 and 4, tube 1 serving as a blank, tube 2 as a control, and tubes 3 and 4 both show the steroid stimulation. The solutions were transferred as quickly as possible to microcurvettes (capacity = 0·3 ml) with 1-cm light paths and the optical densities at 340 mμ were measured continuously at 30-sec intervals for 15–30 min using a Hilger Uvispek spectrophotometer with Gilford automatic recording attachment, which allows each of the four solutions to be measured consecutively. All reaction rates were calculated from the slopes of the initial linear portions of the graphs.

β-Glucuronidase assay

Protein concentration was determined in the extract by measurement of the absorbancies at 280 mμ and 260 mμ and calculations of the protein concentration made by the formula $1{\cdot}5 \times A_{280} - 0{\cdot}75 \times A_{260}$ = protein concentration, mg/ml. The extracts were diluted so that 1 ml of test solution contained about 1 mg of total protein.

The β-glucuronidase estimations were carried out by a modification of the method of Talalay *et al.*[10] Phenolphthalein mono-β-glucuronic acid (0·05 g Sigma) was dissolved in 10 ml ethanol (freshly re-distilled from potassium hydroxide) and diluted to 100 ml with water. The ethanol stabilises the phenolphthalein glucuronic acid in solution.

Enzyme solution (1 ml) acetate buffer (1 ml 0·1 M pH = 4·5) and substrate solution (1 ml) were incubated in a stoppered tube for 1 hr at 37° in a waterbath. A blank containing no enzyme was also incubated. On removal from the waterbath, 1 ml of enzyme solution was added to the blank and glycine buffer (1 ml 0·4 M pH = 10·45) was added to each tube. The tubes were centrifuged at 600 *g* for 15 min and the duplicates read against the blank, using a Unicam SP.600 spectrophotometer at 550 mμ. The activity was expressed in units; 1 unit liberating 1 μg phenolphthalein per hour at 37°.

MATERIALS

6-Mercaptopurine monohydrate was obtained from the California Corporation for Biochemical Research, Los Angeles, U.S.A. Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) was the gift of Lederle Laboratories Division, American Cyanamide Company, Pearl River, N.Y., U.S.A. The ammonium salt of glucosaccharo-1→4-lactone was the gift of Pfizer Ltd., Sandwich, Kent.

RESULTS AND DISCUSSION

The relationship between the growth rate of regenerating liver and the enzyme levels in these tissues is illustrated in Fig. 1. The upper graph shows the weight of regenerating

liver tissue at various periods of time after partial hepatectomy. A group of 36 partially hepatectomised rats were killed in batches of 6 at intervals of 1, 3, 5, 7, 10 and 15 days after operation, the livers were removed and weighed. The weight of regenerated tissue was estimated by subtracting the average weight of liver left in the animal after partial hepatectomy. This weight was obtained from a group of 12 rats which were killed immediately after the operation had been performed.

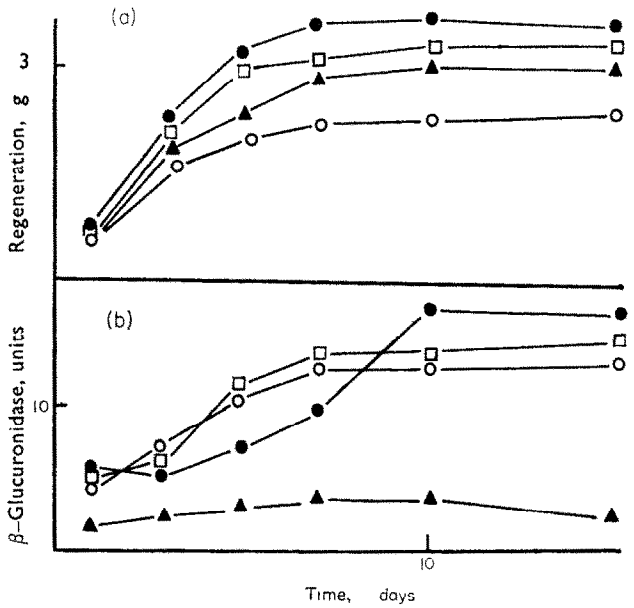

FIG. 1. The effect of drugs on the growth rate and β-glucuronidase activity of regenerating rat liver

(a) The weight of the regenerated portion of rat livers at various times after partial hepatectomy.

(b) The β-glucuronidase activity of regenerating rat liver per mg of liver tissue at various times after partial hepatectomy.

●—● untreated animals
□—□ animals treated with 6-mercaptopurine
○—○ animals treated with methotrexate
▲—▲ animals treated with glucosaccharo-1→4-lactone

Three other groups, each of 36 partially hepatectomised animals (b, c and d) were treated in the same way except that group 2 was given a daily injection of 6-mercaptopurine (1 mg/kg per day, i.p.), group 3 was injected with methotrexate (0·5 mg/kg per day, i.p.) and group 4 treated with glucosaccharo-1→4-lactone (NH_4 salt) which was administered to the animals as a 3% solution in the drinking water. The quantities of antimetabolite used were limited by the toxicity of these substances and the concentration of glucosaccharo-1→4-lactone by the reluctance of the animals to drink water containing more than 5% of this substance. Figure 1a shows that all three of these forms of treatment inhibit to a significant extent the average regeneration of the liver although that produced by either 6-mercaptopurine or glucosaccharo-1→4-lactone is much less than the effect of methotrexate, under these conditions.

The specimens of regenerating liver obtained at various time intervals after partial hepatectomy were perfused *in vivo* with physiological saline containing 25 I.U. of heparin per 100 ml. The specimens were then homogenised and diluted to a suitable concentration and β-glucuronidase estimations were taken on individual specimens. Figure 1b shows the average result obtained from such estimations. In livers from untreated animals the β-glucuronidase levels in liver rise gradually to a maximum at 10 days after operation. It appears surprising that the rise in the enzyme is delayed to this extent as the increase of β-glucuronidase would be expected to bear a direct relationship to the rate of growth of the tissue.

The rise in β-glucuronidase in animals treated with either methotrexate or with 6-mercaptopurine is somewhat earlier than is the case in the untreated animal, reaching a maximum at 6 or 7 days after operation. There appears to be no difference in the response between animals treated with these two drugs. The lower graph shows the effect of treatment with glucosaccharo-1→4-lactone. The slight increase in activity at about 7 days reflects the increase seen in the antimetabolite treated animals but is probably not significant. The variation of the β-glucuronidase appears to bear no direct relationship to the growth rate of the tissue as would be expected if the role of this enzyme were directly associated with metabolic activity.

Figure 2a shows the variation of the 3 α-hydroxysteroid activated nicotinamide

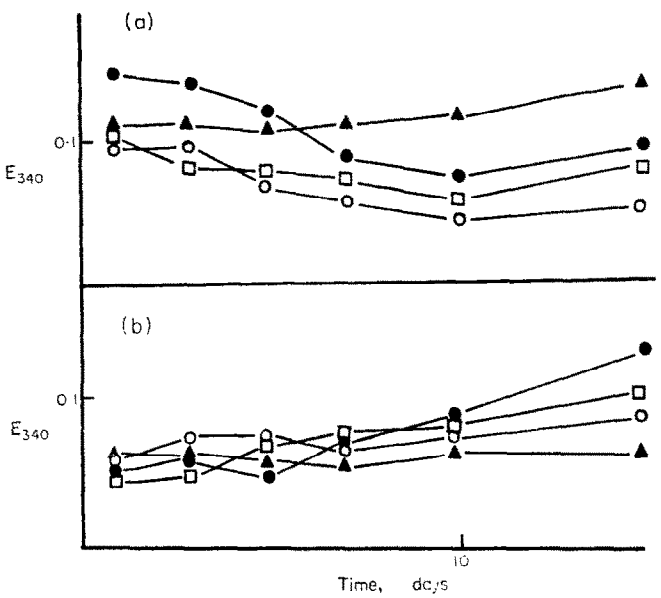

FIG. 2. The effect of drugs on the 3 α-hydroxysteroid activated coenzyme transhydrogenation in regenerating rat liver.

(a) Steroid stimulation of liver extracts at various stages of regeneration.
(b) Background transhydrogenase of liver extracts without the addition of steroid at various stages of regeneration

●—● untreated animals
□—□ animals treated with 6-mercaptopurine
○—○ animals treated with methotrexate
▲—▲ animals treated with glucosaccharo-1→4-lactone

nucleotide coenzyme transhydrogenation during liver regeneration. The homogenised liver extract was partly purified, as described above, and the transhydrogenase levels measured. There is a significant lowering of transhydrogenation of all three methods of treatment relative to the control animals. This effect may be in part due to the *in-vitro* suppression of the enzyme by the drugs or their metabolites, although such an effect seems unlikely in partly-purified extract. There is also a decrease in activity which reaches its lowest level at about 7 days after operation. This decrease is most obvious in untreated animals. It is probably significant that the minimum transhydrogenase levels coincide with increasing β-glucuronidase.

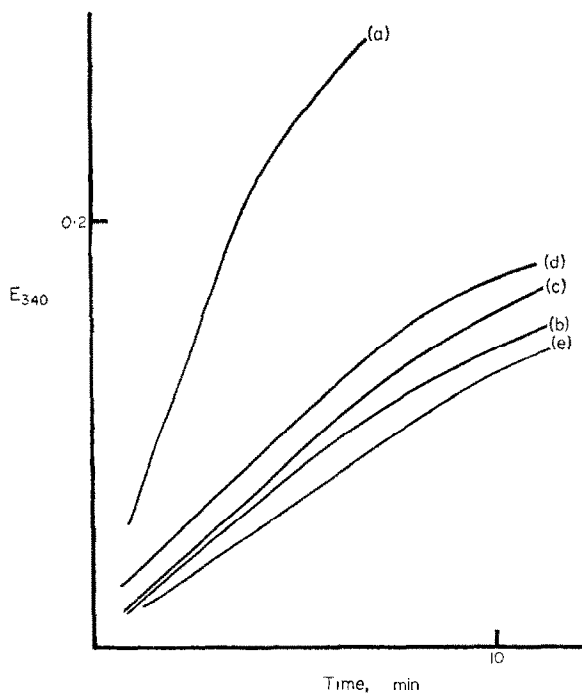

FIG. 3. The effect of previous incubation of crude rat liver extract, from rats 3 days after partial hepatectomy, on the transhydrogenation

(a) The 3 α-hydroxysteroid activated transhydrogenase activity—not incubated.
(b) Control with no steroid added—not incubated.
(c) No steroid added but incubated for 1 hr before estimating the transhydrogenation.
(d) No steroid added but incubated for 3 hr before estimating the transhydrogenation.
(e) No steroid added but incubated for 3 hr with glucosaccharo-1→4-lactone.

The method of estimation used to obtain the transhydrogenase levels (Fig. 2a) gives a result depending upon the transhydrogenation produced by the addition of free steroid hormone and it does not take into account the effect of endogenous steroid already present in the extract. It follows, therefore, that if the endogenous free steroid in the tissue is increased without a change in the overall transhydrogenase activity then there would be an apparent fall in steroid activation. Such a drop is shown in Fig. 2a. Assuming that transhydrogenation plays a vital part in cell growth then an increase in this enzyme would be expected in fast-growing tissue. If, however, the

increased activity is due to available free steroid hormone rather than increased enzyme levels this increase would be reflected in an apparent decrease in the measurable enzyme activity (the difference between activated and background activity).

The background transhydrogenase activity of the regenerating liver is shown in Fig. 2b. The activity is made up of a steroid independent fraction and also a fraction which increases with the release of free steroid into the liver tissue. The quantity of free steroid present in the liver tissue depends on a number of factors including the presence of β-glucuronidase which releases steroids from their conjugated glucuronide form. It is significant that the increase in activity shown in Fig. 2b is similar to the corresponding curves in Fig. 1b which shows the increase of β-glucuronidase activity. The total measurable transhydrogenation can be estimated by the addition of the appropriate curves in Figs. 2a and 2b. The overall activity increases over the period studied in the livers of untreated animals and those having 6-mercaptopurine and methotrexate treatment but not to a significant extent in those with glucosaccharo-1→4-lactone treatment. This indicates that β-glucuronidase plays a part in the increase of transhydrogenation but, of course, part of the effect might be produced by the growth retardation of the liver shown in Fig. 1.

Untreated regenerating liver was taken from animals at the time of maximum and minimum β-glucuronidase content and a crude extract was obtained. The extracts were then incubated for different times with and without glucosaccharo-1→4-lactone. The effect of incubating a crude extract of liver which had been regenerating for 3 days is shown in Fig. 3. In this case the crude liver extract for 1 and 3 hr respectively at 37° and the transhydrogenase activity of the suitably diluted mixture was estimated (curves c and d respectively). Another specimen was incubated with the addition of 5% glucosaccharo-1→4-lactone (curve e). Curves (a) and (b) are comparable curves obtained from the crude preparation without incubation, curve (a) having 1 μg of androsterone added.

Figure 4 shows the curves obtained from an identical experiment using liver which had been regenerating for 10 days as the enzyme source. In this case incubation causes a much greater increase in the background activity which is in agreement with the idea that the β-glucuronidase activity is responsible for much of the increased background activity.

Finally, the enzyme was purified by ammonium sulphate precipitation followed by elution from a DEAE column and heat treatment as above, and incubation experiments were carried out on the purified extracts as shown in Fig. 5. In this figure curves (a) and (b) are the androsterone activated and the control curves as before. Curve (c) now represents 2 hr incubation of the enzyme in the presence of added β-glucuronidase. This addition makes little difference presumably because the conjugated steroids have already been removed by the purification technique. Curve (d) represents similar incubation with 5% glucosaccharo-1→4-lactone and approximately 1 μg of androsterone-3α-glucuronide added and in this case the saccharo-lactone is sufficient to prevent more than a small degree of enzymic hydrolysis of the steroid glucuronide. Curve (e) shows the effect of incubating the enzyme with androsterone glucuronide alone. There is a certain amount of decomposition of the steroid presumably due to the fact that there is still a small β-glucuronidase activity in the purified enzyme. If, however, the enzyme preparation is mixed with a previously incubated mixture of androsterone glucuronide and β-glucuronidase curve (f), a transhydrogenase activity

comparable with that shown in curve (a) is produced. This experiment shows that the transhydrogenase system is not activated by androsterone when this substance is conjugated at the 3 position with glucuronic acid but its capacity to activate the enzyme is restored on incubation with β-glucuronidase.

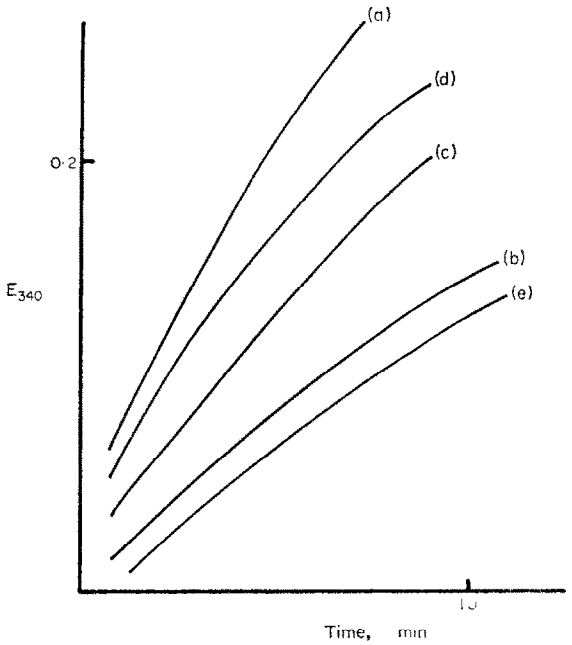

FIG. 4. The effect of previous incubation of crude rat liver extract from rats 10 days after partial hepatectomy on the transhydrogenation

(a) The 3 α-hydroxysteroid activated transhydrogenase activity—not incubated.
(b) Control with no steroid added—not incubated.
(c) No steroid added but incubated for 1 hr before estimating the transhydrogenation.
(d) No steroid added but incubated for 3 hr before estimating the transhydrogenation.
(e) No steroid added but incubated for 3 hr with glucosaccharo-1→4-lactone.

These experiments suggest that β-glucuronidase activity plays an important part in the regeneration of liver tissue after partial hepatectomy, and may have a controlling influence on the balance between free and conjugated steroid in this tissue. In turn the free steroid level in the tissues exerts an influence on the transhydrogenase activity and hence the balance between NAD, NADP and their reduced forms.

The importance of this balance *in vivo* is still not clear but the extremely low steroid concentrations involved in the reaction make it an attractive basis for speculation as to the mechanism by which steroids exert their effect. Various critical examinations of the possible physiological significance of such systems, expecially those of the human placenta have been presented by e.g. Williams-Ashman[11] and Joel, Hagerman and Villee[12] who conclude that transhydrogenation could exert at least some control over

oxidative metabolism. Stein and Kaplan[13] and Bloom,[14] however, conclude that in liver, hydroxysteroid activated transhydrogenation is of minor physiological importance. Much would appear to depend on the local conditions existing within the

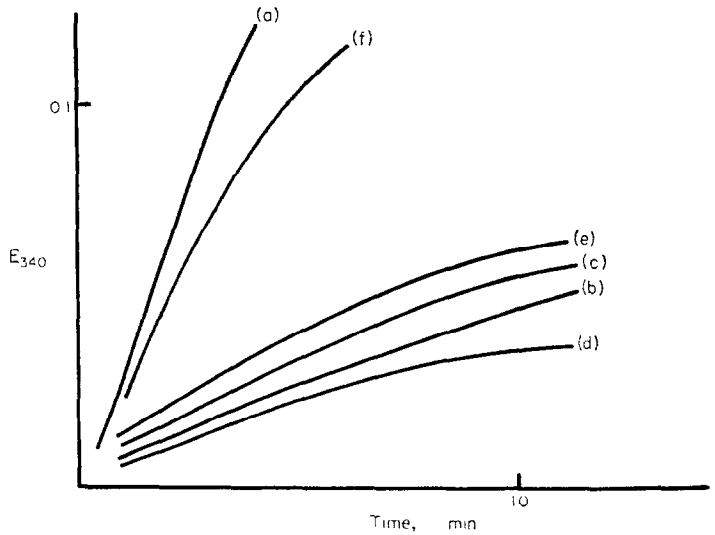

FIG. 5. The effect of previous incubation of purified rat liver extract on the transhydrogenation
(a) 3 α-Hydroxysterid added.
(b) Control—no steroid added.
(c) Control—no steroid added—incubated with β-glucuronidase.
(d) Control with incubated β-glucuronidase, glucosaccharo-1→4-lactone and androsterone glucuronide added.
(e) Control with androsterone glucuronide.
(f) Control with incubated androsterone glucuronide and β-glucuronidase added.

cell and the rapidity of growth of the tissue in question. For this reason this system may well be of more importance in its effect on malignant rather than normal tissue (cf. Hollander *et al.*)[14–15] and also possibly in other rapidly proliferating tissues such as regenerating rat liver.

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REFERENCES

1. G. M. TOMKINS, *J. biol. Chem.* **218**, 437 (1956).
2. B. HURLOCK and P. TALALAY, *J. biol. Chem.* **233**, 886 (1958).
3. D. C. WILLIAMS, D. N. BARON and M. B. R. GORE, *Biochem. J.* **76**, 37P (1960).
4. D. N. BARON, M. B. R. GORE and D. C. WILLIAMS, *Biochem. J.* **74**, 20P (1959).
5. D. N. BARON, M. B. R. GORE and D. C. WILLIAMS, *Act. Endocrinology* **35**, 51, 981 (1960).
6. D. N. BARON, M. B. R. GORE, R. PIETRUSZKO and D. C. WILLIAMS, *Biochem. J.* **88**, 19 (1963).
7. G. T. MILLS, J. PAUL and E. E. B. SMITH, *Biochem. J.* **53**, 245 (1953).
8. R. L. P. ADAMS, *Biochem. J.* **87**, 532 (1963).
9. J. JARABAK, J. A. ADAMS, H. G. WILLIAMS-ASHMAN and P. TALALAY, *J. biol. Chem.* **237**, 345 (1962).

10. P. TALALAY, W. H. FISHMAN and C. HUGGINS, *J. biol. Chem.* **166**, 757 (1946).
11. H. G. WILLIAMS-ASHMAN, *Control Mechanisms in Cellular Processes* O. M. BONNER, Ed., The Ronald Press Co., New York (1961).
12. P. B. JOEL, D. D. HAGERMAN and C. A. VILLEE, *J. biol. Chem.* **236**, 3151 (1961).
13. A. M. STEIN and N. O. KAPLAN, *Science* **129**, 1611 (1959).
14. B. BLOOM, *J. biol. Chem.* **235**, 857 (1960).
15. V. P. HOLLANDER, H. JONAS and D. E. SMITH, *Cancer* **11**, 803 (1958).
16. V. P. HOLLANDER, D. E. SMITH and T. E. ADAMSON, *Cancer* **12**, 135 (1959).