effect on fibril formation *in vitro* does not at the moment appear to have any physiological implications.

SUMMARY

1. Low concentrations of heparin and deoxyribonucleic acid have been found to retard fibril formation in solutions of acid-soluble collagen at pH 7, *I* 0.23 and 25°. Two types of chondroitin sulphate (A and C) and a preparation rich in kerato-sulphate accelerated fibril formation. Another type of chondroitin sulphate (B) and hyaluronic acid had no effect. Those polyanions which accelerated precipitation reduced fibril width and those which retarded precipitation increased it. The accelerating effect of chondroitin sulphate A increased with decreasing pH in the range pH 6–8 and with increasing *I* in the range 0.13–0.31.

2. Chondroitin sulphate A accelerated precipitation only when present during the lag period. This observation supports the conclusion from earlier work that collagen fibrils form as the result of two consecutive processes, nucleation and growth, and it is concluded that chondroitin sulphate A accelerates precipitation by affecting nucleation. Only a very small proportion of the added chondroitin sulphate was incorporated into the collagen precipitate.

3. When collagen solutions were pretreated with phosphate buffer, pH 7, at 0° addition of chondroitin sulphate A retarded subsequent precipitation at 25°, indicating that nucleation had taken place during pretreatment and that the observed retardation was due to the effect of chondroitin sulphate A on growth.

4. The mode of action of the polyanions is considered in the light of the two-step mechanism of fibril formation and the findings are discussed in relation to the possible role of mucopolysaccharides in the formation of collagen fibrils *in vivo*.

The author wishes to thank Dr M. K. Keech for co-operating in the electron-microscopy aspects of the work; Mr A. Field for technical assistance; Dr D. A. Hall and Professor R. E. Tunbridge for their generous support during the work and for many stimulating discussions. Thanks are also due to Dr P. F. Lloyd for supplying mucopolysaccharide preparations from bovine intervertebral disk, to Professor K. Meyer for samples of chondroitin sulphates and to Dr K. Kirby and Dr P. W. Kent for gifts of nucleic acid preparations.

REFERENCES

- Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
 di Ferrante, N. (1956). *J. biol. Chem.* **220**, 303.
 Dunphy, J. E. & Udupa, K. N. (1955). *New Engl. J. Med.* **253**, 847.
 Gross, J. (1956). *J. biophys. biochem. Cytol.* **2**, 261.
 Gross, J. (1959). *Connective Tissue, Thrombosis and Atherosclerosis*, p. 77. Ed. by Page, I. H. New York: Academic Press Inc.
 Gross, J. & Kirk, D. (1958). *J. biol. Chem.* **233**, 355.
 Hall, D. A., Lloyd, P. F., Happey, F., Horton, W. G. & Naylor, A. (1957). *Nature, Lond.*, **179**, 1078.
 Hodge, A. J. & Schmitt, F. O. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 418.
 Jackson, S. F. (1955). *Proc. Roy. Soc. B*, **144**, 556.
 Kühn, K., Grassmann, W. & Hofmann, U. (1957). *Naturwissenschaften*, **44**, 538.
 Kühn, K., Hofmann, U. & Grassmann, W. (1959). *Naturwissenschaften*, **46**, 512.
 Meyer, K. (1946). *Amer. J. Med.* **1**, 675.
 Meyer, K. (1955–56). *Harvey Lect.* **51**, 97.
 Meyer, K., Linker, A., Davidson, E. A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
 Randall, J. T., Booth, F., Burge, R. E., Jackson, S. F. & Kelly, F. C. (1955). *Symp. Soc. exp. Biol.* **9**, 127.
 Van den Hooff, A. (1957). *Counc. int. Org. Med. Sci. Symp.: Connective Tissues*. Ed. by Tunbridge, R. E. Oxford: Blackwell Scientific Publications Ltd.
 Wood, G. C. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, p. 26, 2–92.
 Wood, G. C. (1960). *Biochem. J.* **75**, 598.
 Wood, G. C. & Keech, M. K. (1960). *Biochem. J.* **75**, 588.

Biochem. J. (1960) **75**, 612

Steroid Sulphatase, Arylsulphatase and β -Glucuronidase in the Mollusca

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(Received 9 November 1959)

In recent years considerable interest has been shown in the use of enzymes from molluscan tissues for the hydrolysis of urinary steroid conjugates (Henry & Thevenet, 1952; Stitch & Halkerton, 1953*a, b*, 1956; Jayle & Baulieu, 1954).

Although these tissues are known to be rich sources of β -glucuronidase and arylsulphatase (Dodgson, Lewis & Spencer, 1953) and to possess steroid sulphatases, the specificity of the latter has been investigated in only two species. An enzyme

present in extracts of *Otala punctata*, a tropical terrestrial gastropod, was reported by Savard, Bagnoli & Dorfman (1954) to hydrolyse dehydro-epiandrosterone sulphate but not the sulphates of androsterone or testosterone. Roy (1954, 1956a) prepared a highly active concentrate of steroid sulphatase from an acetone-dried powder of *Patella vulgata* and showed that the enzyme was specific for the 3β -sulphates of the 5α and Δ^5 steroids, but had no action on the other isomeric 3 -sulphates.

The specificity of the steroid sulphatases from these two gastropods limits their usefulness in the hydrolysis of urinary steroid conjugates because both 3α -hydroxy- 5α and -5β steroids may be excreted as sulphates. On the other hand, hydrolysis of conjugates with strong acid at higher temperatures leads to the formation of artifacts from some steroids, and the use of milder conditions, such as continuous ether extraction at pH 1, is time-consuming. These factors prompted a search for a convenient source of enzyme capable of hydrolysing androsterone sulphate, and the present study has been primarily concerned with an investigation of the steroid sulphatases present in various marine molluscs. Because many of the animals used in this work had not been investigated as possible sources of arylsulphatase and β -glucuronidase, it was considered worth while to determine whether these enzymes could be detected in each of the species examined, and whether their activities were in any way correlated with those of the steroid sulphatases.

MATERIALS AND METHODS

Extraction of enzymes from the test animals

Most of the species were collected near Plymouth Sound. They were dissected as soon as they had been brought into the laboratory and were used for the preparation of an acetone-dried powder, of the kind which Dodgson & Spencer (1953) have described as powder A. Usually the tissue chosen for study was the digestive gland (hepatopancreas); when the test material was too small for this tissue to be dissected, whole animals, including their shells, were ground in a pestle and mortar and the debris obtained was used in the preparation of powder A.

Substrates

p-Chlorophenyl glucuronide monohydrate was prepared by the method of Spencer & Williams (1951), dipotassium 2-hydroxy-5-nitrophenyl sulphate by the method of Roy (1956a), and steroid sulphates by the method of Roy (1956a). A pure sample of *p*-nitrophenyl sulphate was obtained from Sigma Chemical Company, Missouri, U.S.A.

Measurement of enzymic activity

β -Glucuronidase. This was assayed by the method of Dodgson *et al.* (1953), in 0.5M-sodium acetate-acetic acid buffer, pH 4. The pH optimum of 4.0 reported by these

workers for the hydrolysis of *p*-chlorophenyl glucuronide with tissue homogenates of *P. vulgata* and *Littorina littorea* was used throughout, but the optimum substrate concentration for the powder prepared from the visceral hump of *P. vulgata* was redetermined because an acetone-dried powder was used instead of a homogenate. As only a small increase in enzymic activity was detected with substrate concentrations greater than 0.045M (Fig. 1), this concentration of *p*-chlorophenyl glucuronide was used in all the assays of β -glucuronidase. All the estimations of enzymic activity were made with a final concentration of 400 μ g. of the powder obtained from each species/ml.

Arylsulphatase. This was assayed with 2-hydroxy-5-nitrophenyl sulphate as the substrate by the method of Dodgson & Spencer (1953), as modified by Dodgson *et al.* (1953). The pH optimum for the hydrolysis of 2-hydroxy-5-

Fig. 1. Effect of substrate concentration on β -glucuronidase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 0.8 ml., comprising 0.4 ml. of enzyme solution in 0.5M-acetate buffer, pH 4.0, and 0.4 ml. of *p*-chlorophenyl glucuronide solutions of various concentrations in the same buffer. Incubation was for 1 hr. at 37.5°.

Fig. 2. Effect of substrate concentration on arylsulphatase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 1.2 ml., comprising 0.6 ml. of enzyme solution in 0.5M-acetate buffer, pH 5.5, and 0.6 ml. of nitrocatechol sulphate solutions of various concentrations in the same buffer. Incubation was for 1 hr. at 37.5°.

nitrophenyl sulphate with the arylsulphatase of *P. vulgata* has been reported by Roy (1956a) to be 5.4–5.6, and a pH of 5.5 was therefore used in the present study. As the enzyme prepared by Roy was highly purified, it was necessary to determine the optimum substrate concentration for the crude powder from *P. vulgata*. At pH 5.5, in acetate buffer, this concentration was found to be 12 mM (Fig. 2). Measurements of enzymic activity were made on 100 μ g. of the powder obtained from each species/ml.

Assays of enzymic activity were also carried out with *p*-nitrophenyl sulphate as the substrate and the method used was similar to that described above, except that the liberated *p*-nitrophenol was read at 400 m μ . The pH optimum for the powder prepared from *P. vulgata* was found to be 5.5–5.7 (Fig. 3) and, at pH 5.5, the optimum substrate concentration was 0.03 M (Fig. 4). The assays were carried out in 0.5 M-sodium acetate-acetic acid buffer, pH 5.5.

Fig. 3. Effect of pH on arylsulphatase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 1.2 ml. comprising 0.3 ml. of enzyme solution, 0.3 ml. of 2.0 M-acetate buffers of various pH values, and 0.6 ml. of 0.06 M-*p*-nitrophenyl sulphate. Incubation was for 1 hr. at 37.5°.

Fig. 4. Effect of substrate concentration on arylsulphatase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 1.2 ml., comprising 0.6 ml. of enzyme solution in 0.5 M-acetate buffer, pH 5.5, and 0.6 ml. *p*-nitrophenyl sulphate solutions of various concentrations in the same buffer. Incubation was for 1 hr. at 37.5°.

Steroid sulphatase. This was assayed by the method of Roy (1956a) with the diluted methylene-blue reagent described by Roy (1956b). The absorption of the steroid sulphate-methylene blue complex was measured at 655 m μ (Unicam SP. 400 spectrophotometer). Each enzyme preparation was tested at six different pH values in 0.5 M-sodium citrate-HCl buffer (pH 3.0), 0.5 M-sodium acetate-acetic acid buffers (pH 4.0, 5.0 and 6.0) and 0.5 M-2-amino-2-hydroxymethylpropane-1:3-diol-acetic acid buffers at pH 7.0 and 8.0. The final concentration of the buffers in the assays was 0.125 M after addition of the substrate. The partition of the steroid sulphate-methylene blue complex between the aqueous and chloroform phases was not influenced by pH in the range 4.0–8.0, but at pH 3.0 it increased by about 5% in favour of the chloroform phase. Two controls were therefore used in each experiment, one at pH 3.0 and the second at one of the other pH values. A typical example of the effect of pH on the activity of steroid sulphatase is shown in Fig. 5.

Incubations were carried out at 37° for 2 hr. with androsterone sulphate and dehydroepiandrosterone sulphate (DHAS) as the substrates; and at 45° for 17 hr. with aetiocholanolone sulphate as substrate. The latter conditions were suggested by Dr I. E. Bush (personal communication). Certain species were also tested for androsterone sulphate activity under these conditions. In addition, if hydrolysis (1–2%) of androsterone sulphate or DHAS took place after 2 hr. the assay was repeated with a 17 hr. incubation period to check whether or not the results of the first assay were significantly different from zero. All three substrates are stable under these conditions between pH 3.0 and 8.0. The effect of increasing the enzyme concentrations from 0.3 to 3.0 mg./ml. on the hydrolysis of DHAS was studied with the powder prepared from *Helix pomatia* crop fluid. Enzymic activity increased linearly up to a concentration of 0.9 mg./ml. and then curved asymptotically. Enzymic activity was assayed on 1.5 mg. of the powder prepared from each test species/ml.

Fig. 5. Effect of pH on steroid-sulphatase activity of *H. pomatia* crop fluid. The volume of the reaction mixture was 2.0 ml., comprising 0.5 ml. of enzyme solution, 1.0 ml. of DHAS solution, and 0.5 ml. of 0.5 M buffers of various pH values. Incubation was for 2 hr. at 37°.

RESULTS

The activities of steroid sulphatase, arylsulphatase and β -glucuronidase from various marine molluscs are summarized in Table 1. *H. pomatia*, the only terrestrial species, has been included for comparison because this animal is commonly used as a source of enzymes for the hydrolysis of steroid conjugates.

The values shown for arylsulphatases and β -glucuronidase were derived from duplicate estimations, and for steroid sulphatases from single estimations, of the acetone-dried powder prepared from each species. The possible influence of factors such as season and sex on enzymic activity was not taken into account. In addition, arylsulphatases and β -glucuronidase were assayed with the pH optima and optimum substrate concentrations found for *P. vulgata*, and no allowance was made for possible variation among the different species. However, the optimum pH found for the hydrolysis of *p*-nitrophenyl sulphate in this study was the same as that reported by Roy (1956*a*) for a much purer enzyme preparation from *P. vulgata* with 2-hydroxy-5-nitrophenyl sulphate as substrate; it also agrees with the value found by Dodgson *et al.* (1953), for homogenates of tissues from *P. vulgata* and *L. littorea* and potassium *p*-acetylphenyl sulphate as substrate. It is therefore possible that there is little variation in pH optima for the hydrolysis of arylsulphates by the enzymes prepared from molluscan tissues. Dodgson *et al.* (1953) reported results for the arylsulphatase and β -glucuronidase present in tissue homogenates prepared from *P. vulgata*, *L. littorea* and *Buccinum undatum*. The results of the β -glucuronidase assays in Table 1 (when converted into units/g. of tissue) are all within the ranges reported by the above workers. Arylsulphatase values cannot be compared because of the different substrates used.

The optimum pH of 4.0–5.0 for the hydrolysis of DHAS by acetone-dried powders prepared from gastropods is in agreement with the pH optimum reported by Savard *et al.* (1954) for *O. punctata* (pH 4.5–5.0), and that reported by Roy (1954) for *P. vulgata* (pH 4.5). Roy (1956*a*), using the steroid sulphatase of *P. vulgata*, has also reported the optimum substrate concentrations for the hydrolysis of DHAS (0.2 mM), *epi*androsterone sulphate (> 0.2 mM) and pregnenolone sulphate (0.06–0.08 mM). As might be expected, the optimum substrate concentration varies with the substrate used. The concentrations of steroid sulphates used in this study (Table 1) were not optimum but were sufficiently high to detect whether or not steroid sulphatases were present.

Attempts were made to increase the rate of hydrolysis of DHAS by the enzyme from *H. pomatia*

crop fluid by using possible activators. The influence of two compounds which are known to enhance the activity of β -glucuronidase was investigated. Albumin had no effect, and propylene glycol [which acts as a glucuronic acid acceptor in enhancing β -glucuronidase activity (Fishman & Green, 1956)], depressed the steroid-sulphatase activity by 47%. Barium chloride, which might be expected to increase the rate of hydrolysis of sulphates by removal of sulphate ions, had no effect.

DISCUSSION

The present study has confirmed previous observations on the widespread occurrence of arylsulphatase and β -glucuronidase among marine molluscs. From this survey, however, it appears that steroid sulphatase is confined to one class of the molluscs, the gastropods, and even in this class steroid sulphatase is not invariably found. No correlation could be found between the activity of the arylsulphatases and steroid sulphatases, and neither of these activities could be correlated with that of β -glucuronidase.

Various terms have been used to describe the enzyme present in *P. vulgata* which hydrolyses DHAS. Stitch & Halkerston (1953*a*) describe the enzyme as an 'alkyl sulphatase', whereas Roy (1956*a*) uses the more specific term ' β -steroid sulphatase'. The present study of steroid sulphatases shows that the very high specificity of the enzyme from *P. vulgata* is not found in all gastropods. Three of the species studied were shown to possess enzymes which could hydrolyse aetiocholanolone sulphate as well as DHAS, but an enzyme which would hydrolyse androsterone sulphate was not detected. Species possessing aetiocholanolone sulphate activity (*H. pomatia*, *B. undatum* and *Nassarius reticulatus*) were those which showed the highest specific activity with respect to the hydrolysis of DHAS. This raises the question whether the same enzyme acts on both substrates, DHAS being more readily hydrolysed because of its structural configuration. If this were so, it might be expected that the hydrolysis of androsterone sulphate could only be achieved by an enzyme having very high steroid-sulphatase activity. However, it was found that a powder D (Dodgson & Spencer, 1953), prepared from *P. vulgata*, would not hydrolyse aetiocholanolone sulphate, although possessing an activity three times that of powder A with respect to the hydrolysis of DHAS. Moreover, the highly purified preparation of *P. vulgata* used by Roy (1956*a*) would not hydrolyse aetiocholanolone sulphate. These findings are consistent with the view that two separate enzymes are involved in the hydrolysis of DHAS and aetiocholanolone sulphate, but

Table 1. *Arylsulphatase, β -glucuronidase and steroid-sulphatase activities of various molluscs*

Species	Source of enzyme	Arylsulphatase			β -Glucuronidase	Steroid sulphatase at pH							
		NCS	NPS			3	4	5	6	7	8		
<i>Helix pomatia</i>	Digestive gland	760	367		231	AeS DHAS	0 0	1 206 891	2 261 1 400	301 1 273	301 891	0 891	
	Crop fluid*	2551	823		58	AeS DHAS	904 1 018	1 507 3 946	8 138 8 147	2 562 3 055	2 261 1 782	0 0	
<i>Patella vulgata</i>	Visceral hump	652	157		156	AeS DHAS	0 0	0 1 006	0 2 012	0 1 868	0 862	0 431	
<i>Littorina littorea</i>	Whole animal less shell	586	169		69	AeS DHAS	0 509	0 891	0 637	0 382	0 0	0 0	
<i>Buccinum undatum</i> *	Digestive gland	228	81		15	AeS DHAS	1 055 431	6 631 3 592	12 056 2 299	1 808 1 581	754 862	301 0	
<i>Aplysia punctata</i> *	Digestive gland	287	55		29	AeS DHAS	0 0	0 0	0 0	0 0	0 0	0 0	
<i>Nucella lapillus</i> *	Digestive gland	96	39		15	AeS DHAS	0 0	0 2 465	0 2 900	0 1 015	0 1 015	0 1 015	
<i>Nassarius reticulatus</i> *	Digestive gland	436	236		76	AeS DHAS	2 230 5 250	10 409 10 650	6 394 10 650	595 2 700	466 1 200	0 0	
<i>Patina pellucida</i>	Whole animal	26	7		24	AeS DHAS	0 0	0 450	0 900	0 900	0 750	0 0	
<i>Turritella communis</i>	Whole animal	181	37		0	AeS DHAS	0 0	0 0	0 0	0 0	0 0	0 0	
<i>Crepidula fornicata</i>	Digestive gland	74	9		8	AeS DHAS	— 0	— 0	— 0	— 0	— 0	— 0	
<i>Pecten maximus</i>	Digestive gland	941	449		17	AeS DHAS	0 0	0 0	0 0	0 0	0 0	0 0	
<i>Sepia officinalis</i>	Digestive gland	59	19		10	AeS DHAS	— 0	— 0	— 0	— 0	— 0	— 0	
<i>Lepidochitona cinerea</i>	Whole animal	41	25		11	AeS DHAS	0 0	0 0	0 0	0 0	0 0	0 0	

species marked *; no activity was detected.

attempts to confirm this by separating the enzymes by starch electrophoresis have been unsuccessful. In this paper the term 'steroid sulphatase' is used to describe any enzyme which will hydrolyse a steroid sulphate. The use of this term is not intended to imply that the physiological role of these enzymes in the Mollusca is to hydrolyse steroid sulphates, as their function is at present obscure.

From the data of Kellie & Wade (1957) it can be calculated that, of the urinary 17-oxo steroid fraction which is not hydrolysed when treated with β -glucuronidase, but which is hydrolysed when the urine is continuously extracted with ether at pH 1 for 72 hr., 70% is dehydroepiandrosterone, 20% is androsterone and 10% is aetiocholanolone. Presumably this fraction consists of steroids conjugated with sulphuric acid. Further, androsterone sulphate has been isolated from the urine of a patient with an interstitial-cell tumour of the testis (Venning, Hoffman & Browne, 1942). Thus enzymic hydrolysis of all the steroid sulphates present in human urine cannot be achieved with any of the enzyme sources studied in this work. In particular, androsterone sulphate is unaffected, and this possibly means that 3 α -sulphates of other 3 α :5 α compounds are not hydrolysed either. The use of enzyme preparations from *P. vulgata* in the quantitative determination of urinary 17-oxo steroids (see Stitch & Halkerston, 1956) cannot be recommended, since these will not hydrolyse androsterone sulphate or aetiocholanolone sulphate. Moreover, it is unlikely that sulphatases of fungal and bacterial origin would hydrolyse the neutral 17-oxo steroids because mylase P and Takadiastase (Cohen & Bates, 1949; Stitch & Halkerston, 1953b) and microbiological sulphatases (Buehler, Katzman & Doisy, 1950) are ineffective. The steroid sulphatases prepared from mammalian sources, e.g. rat and ox liver, have the same specificity requirements as those of the enzyme from *P. vulgata*, but exhibit a much lower activity with respect to the hydrolysis of DHAS (Gibian & Bratfisch, 1956; Roy, 1957).

The most convenient marine sources of enzymes for use in analytical work with conjugated steroids seem to be *B. undatum* and *N. reticulatus*, for preparations made from these animals hydrolyse both aetiocholanolone sulphate and DHAS and possess considerable β -glucuronidase and arylsulphatase activity. However, the sulphatases have no measurable influence on the hydrolysis of androsterone sulphate and a suitable source of material capable of effecting this process has yet to be found. Preliminary results on species from invertebrate phyla other than the Mollusca (Corner, Leon & Bulbrook, 1960) indicate that steroid sulphatase is absent.

SUMMARY

1. Acetone-dried powders prepared from thirteen species of molluscs have been tested for steroid-sulphatase activity with the sulphates of dehydroepiandrosterone, androsterone and aetiocholanolone as substrates.

2. Three species (*Helix pomatia*, *Buccinum undatum* and *Nassarius reticulatus*) were found to possess activity towards both dehydroepiandrosterone sulphate and aetiocholanolone sulphate, whereas in four species (*Patella vulgata*, *Littorina littorea*, *Patina pellicida* and *Nucella lapillus*) activity towards only dehydroepiandrosterone sulphate was found. Steroid-sulphatase activity was not detected in six species.

3. No enzyme capable of hydrolysing androsterone sulphate was found in any of the animals tested; therefore complete enzymic hydrolysis of urinary steroid conjugates cannot be achieved with enzyme preparations from these molluscs.

4. β -Glucuronidase and arylsulphatase activity are widely distributed in the Mollusca, but the activities of these enzymes are not related to the activity of steroid sulphatase.

We are grateful to Dr W. Klyne for generous gifts of steroids from the M.R.C. Reference Collection, to Dr A. B. Roy and Dr I. E. Bush for helpful advice, and to Mr G. Dean for his technical assistance. We are also very much indebted to Mr P. C. Williams for his help in preparing the *p*-chlorophenyl-glucuronide.

REFERENCES

- Buehler, H. J., Katzman, P. A. & Doisy, E. A. (1950). *Fed. Proc.* **9**, 157.
- Cohen, H. & Bates, R. W. (1949). *Endocrinology*, **44**, 317.
- Corner, E. D. S., Leon, Y. A. & Bulbrook, R. D. (1960). *J. Mar. biol. Ass. U.K.* **39**, 51.
- Dodgson, K. S., Lewis, J. I. M. & Spencer, B. (1953). *Biochem. J.* **55**, 253.
- Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
- Fishman, W. H. & Green, S. (1956). *J. Amer. chem. Soc.* **78**, 880.
- Gibian, H. & Bratfisch, G. (1956). *Hoppe-Seyl. Z.* **305**, 265.
- Henry, R. K. & Thevenet, M. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 896.
- Jayle, M. F. & Baulieu, E. E. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1391.
- Kellie, A. E. & Wade, A. P. (1957). *Biochem. J.* **66**, 196.
- Roy, A. B. (1954). *Biochim. biophys. Acta*, **15**, 300.
- Roy, A. B. (1956a). *Biochem. J.* **62**, 41.
- Roy, A. B. (1956b). *Biochem. J.* **63**, 294.
- Roy, A. B. (1957). *Biochem. J.* **66**, 700.
- Savard, K., Bagnoli, E. & Dorfman, R. I. (1954). *Fed. Proc.* **13**, 289.
- Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
- Stitch, S. R. & Halkerston, I. D. (1953a). *Nature, Lond.*, **172**, 398.
- Stitch, S. R. & Halkerston, I. D. (1953b). *J. Endocrin.* **9**, xxxvi.
- Stitch, S. R. & Halkerston, I. D. (1956). *Biochem. J.* **63**, 710.
- Venning, E. H., Hoffman, M. M. & Browne, J. S. L. (1942). *J. biol. Chem.* **146**, 369.