Journal of Steroid Biochemistry, 1978, Vol. 9, pp. 1093–1097. Pergamon Press. Printed in Great Britain

ACIDIC STEROID METABOLITES: TRITIUM TRANSFER AND THE *IN VITRO* FORMATION OF 4-PREGNENE-3,20-DIONE-21-OIC ACID BY RABBIT AND RAT HEPATIC AND EXTRAHEPATIC TISSUES

IAN R. SENCIALL and ARUN C. DEY
Medical School, Health Sciences Complex, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3V6

(*Received* 9 *February* 1978)

SUMMARY

Rat and rabbit tissue homogenates detritiated 21-[^{3}H]-deoxycorticosterone when incubated *in vitro*. The adrenal was more active than the liver in both species. Acid formation, as assessed by detritiation, was greater than the net acids obtained by solvent partition. Rabbit adrenal had 78% of the activity of the liver when measured by the latter method, followed by the ovary and adrenal with 57.3% and 51.8%. Rat tissues had less than 50% the activity of the liver. Tritiated 4-pregnene-3,20-dione-21-oic acid (pregnenoic acid*) and 4-androstene-3-one-17β-carboxylic acid (etienic acid) were identified as *in vitro* metabolites of deoxycorticosterone after incubation with rat and rabbit adrenal, ovary, uterus, muscle and kidney homogenates. The possible widespread oxidative metabolism of the corticosteroid side-chain to steroidal-21-oic acids and further to C-20-acids, by the extrahepatic tissues of these two species is indicated.

INTRODUCTION

Recent studies from this laboratory have shown that the rabbit liver microsomal fraction is active *in vitro* in the 21-hydroxylation of P to DOC [1] and the oxidative metabolism of DOC to 21-oic acids [2–6]. Several steroidal-21-oic acids have also been isolated from urine [2, 7, 8].

The oxidative metabolism of the corticoid side-chain to 21-oic acids constitutes a new and important pathway of steroid metabolism that has been shown to occur with several mammalian species [9–15]. The majority of the *in vitro* studies on the formation of steroidal-21-oic acids have centered on the liver which is the principal metabolising organ [1, 3, 6, 9, 12–14] with occasional studies on the adrenal and other tissues [16, 17].

The present study compares the *in vitro* formation of acidic metabolites by several rat and rabbit tissues and demonstrates the extrahepatic biosynthesis, albeit in low yield, of a steroidal-21-oic-acid.

METHODS

Tritium transfer experiments

Synthesis of [21-^{3}*H*]-*deoxycorticosterone.* 21-Dehydro DOC was synthesised by oxidation of DOC with cupric acetate [18]. It was reduced to [21-^{3}H]-deoxycorticosterone by reaction with sodium borotritiide, as described by Willingham and Monder [19]. The product was purified on a silica gel column (15 × 4 cm; mesh 100–200; Nutritional Biochemical Co., U.S.A.) and eluted with chloroform–methanol (95:5, v/v). t.l.c. in the system chloroform–ethyl acetate (8:2, v/v) on Anasil silica gel GF_{254} plates (0.25 mm thickness; Analabs Inc., U.S.A.) gave a single peak of radioactivity that co-chromatographed with authentic DOC (R_F 0.47). An aliquot was also crystallised to constant specific activity with DOC. The specific activity of [21-^{3}H]-DOC was estimated with the blue tetrazolium colorimetric assay to be 27 mCi/mmol.

Preparation of tissue incubates. Groups of five female Sprague–Dawley rats were individually sacrificed with chloroform and samples of tissues were pooled. Groups of two female New Zealand White rabbits were sacrificed by a blow on the neck and tissues were also pooled. Homogenates were prepared in the sucrose–phosphate buffer mixture (pH 7.4) previously described [3], with 0.1 g tissue/ml buffer (10% w/v) being taken with the ovaries and adrenals and 2.0 g tissue/10 ml buffer (20% w/v) with the uterus, muscle, liver, lung, brain and kidney. The homogenates were centrifuged at 2,500 rev./min for 10 min and the clear supernatant was decanted and analysed for protein [20].

Incubation of homogenate supernatants. Approximately 100,000 d.p.m. of 21-[^{3}H]-DOC (554.4 ng; 1.67 nmol) was dissolved in propylene glycol (0.2 ml)

* The following trivial names and abbreviations are used: P; progesterone (4-pregnene-3,20-dione); DOC; deoxycorticosterone (21-hydroxy 4-pregnene-3,20-dione); 21-dehydro-DOC (4-pregnene-3,20-dione-21-al); pregnenoic acid (4-pregnene-3,20-dione-21-oic acid); etienic acid (4-androstene-3-one-17β-carboxylic acid).

and incubated with the tissue homogenate supernatants at a protein:substrate ratio of 20,000:1. A NADPH-generating system [3] was added to each incubate and the total volume was adjusted to 2 ml with bufffer. Incubations were shaken in a water bath at 37°C for 2 h in an atmosphere of air.

Distillation of tritiated water. Incubations were terminated by freezing in an acetone-solid carbon dioxide bath, then stored at −17°C until processed. The water in each incubate was distilled under reduced pressure through an all glass manifold and the distillate was condensed in a flask surrounded by an acetone-solid carbon dioxide bath. One millilitre aliquots of the distillate were counted for radioactivity.

Tissue homogenate incubations with [1,2-^3H]-deoxycorticosterone and [1,2-^3H]-progesterone. Low speed tissue homogenate supernatants (2,500 rev./min) were prepared from the liver, kidney, uterus, ovary, adrenal and skeletal muscle of the rat and rabbit as described for the detritiation experiments. The supernatants were incubated at 20,000:1 protein:substrate ratio with either 1,2-[^{3}H]-DOC (1.86×10^6 d.p.m.; 5.22 ng) or 1,2-[^{3}H]-P (1.97×10^6 d.p.m.; 6.13 ng) in the presence of a NADPH-generating system [3].

Steroid acids were isolated from scaled up incubations of 1,2-[^{3}H]-DOC (11.1×10^6 d.p.m.; 31.2 ng) with the total low speed (2,500 rev./min) supernatants prepared from 10% w/v homogenates of ovary and adrenal (0.35 and 0.4 g wet wt. respectively) and 25% w/v homogenates of liver, kidney, uterus and muscle (2.5 g wet wt. of each). Incubations were carried out as described for the detritiation experiments, except that NADPH was omitted after it was found to have no stimulatory effect on acid formation, the homogenates presumably having sufficient endogenous concentrations of co-factors.

Incubations were terminated by addition of ethyl acetate (5 ml). Radiometabolites were extracted into ethyl acetate at pH 1 and partitioned into neutral and acidic fractions as previously described [11].

Thin layer chromatography. Acidic metabolites and their methyl ester derivatives [5] were chromatographed on Anasil GF_{254} coated plates (0.25 mm thickness; analabs, Inc., U.S.A.) or pre-coated NEN-Silica Gel (OF) plates (New England Nuclear (Canada) Ltd., 0.25 mm thickness).

The following thin layer systems were used: System 1. Ethyl acetate–isopropyl alcohol–formic acid (80:20:1, by vol.; System 2. Chloroform–ethyl acetate (95:5 v/v); System 3. Methyl cyclohexane–ethyl acetate–formic acid (50:50:1, by vol).

Tritium labelled metabolites were detected on thin layer chromatograms with a radiochromatogram scanner (Packard Instruments, Model 3001). Pregnenoic and etienic acids synthesised as previously described [6] were run as internal standards during TLC and were detected by their U.V. light absorption at 240 nm.

Radioactivity. [1,2-^{3}H]-deoxycorticosterone ([1,2-^{3}H]-DOC) (46.8 Ci/mmol); [1,2-^{3}H]-progesterone ([1,2-^{3}H]-P) (55.7 Ci/mmol) and sodium borotritiide (204.8 mCi/mmol) were purchased from New England Nuclear (Canada) Ltd. Aqueous samples (0.5–1.0 ml) were counted in Insta-gel scintillation fluid (10 ml; Packard Instruments Ltd., U.S.A.), and non-aqueous samples in a PCS-xylene scintillation fluid (Amersham-Searle, U.S.A.) as previously described [3]. Counting efficiencies for tritium ranged from 30–35%.

RESULTS

Detritiation of [21-^3H]-deoxycorticosterone by rabbit and rat tissues

During the oxidation of [21-^{3}H]-DOC to the steroidal-21-oic acid there is a transfer of tritium to water [13, 16]. 21-[^{3}H]-DOC was incubated with rat and rabbit homogenates for 2 h as described in the Methods and the incorporation of tritium into water was assayed by distilling the water under reduced pressure. Table 1 compares the detritiation activity of rat and rabbit tissues with that of the liver. All the tissues examined had activities greater than the blank experiments incubated in the absence of tissue, which averaged 4.6% of the liver. The adrenal tissue from both species exhibited greater detritiation activity than the liver.

Table 1. Metabolic transfer of tritium from [21-^{3}H]deoxycorticosterone to water by rat and rabbit tissue homogenates

	d.p.m. ^{3}H/2 ml water distillate* Percentate of liver detritiation activity	
Tissue	Rabbit	Rat
Liver	100.0	100.0
Adrenal	205.0	113.0
Ovary	54.4	60.6
Uterus	38.4	11.4
Lung	31.2	26.0
Kidney	66.7	15.6
Muscle	85.5	39.0

* Mean of duplicate estimations on two experiments.

Table 2. Percentage conversion* of [1,2-^{3}H]-progesterone and [1,2-^{3}H]-deoxycorticosterone to tritiated acids by rat and rabbit tissue homogenates

Tissue	Rat		Rabbit	
	P	DOC	P	DOC
Liver	16.2 ± 0.3	18.8 ± 7.0	13.2 ± 0.9	16.4 ± 0.8
Adrenal	4.6 ± 1.4	5.8 ± 0.4	8.0 ± 0.2	12.9 ± 0.0
Ovary	7.1 ± 0.3	7.2 ± 4.1	4.1 ± 0.6	9.4 ± 0.8
Uterus	5.5 ± 0.3	6.4 ± 1.3	6.1 ± 0.0	8.5 ± 1.5
Muscle	6.7 ± 1.9	6.1 ± 1.1	5.2 ± 0.8	4.0 ± 0.9
Lung	4.3 ± 0.3	3.8 ± 0.4	5.0 ± 1.2	6.4 ± 0.7
Kidney	3.4 ± 0.2	3.9 ± 0.3	4.1 ± 0.1	4.8 ± 0.6

* Mean of duplicate estimations on two experiments.

Formation of [^{3}H]-acids from ring A tritiated progesterone and deoxycorticosterone

[1,2-^{3}H]-DOC and [1,2-^{3}H]-P were incubated with rat and rabbit tissue homogenates as described in the Methods, under the same conditions as the detritiation experiments. The formation of acids was assessed by solvent partition. Table 2 gives the percentage formation of acids by both rat and rabbit tissues. Several differences were noted when the results of the detritiation experiments were compared with those involving the assessment of acid formation by solvent partition. In general, the estimation of acid formation by the latter procedure was less than by detritiation activity. This was particularly marked with the adrenals of the rabbit and rat where the activities relative to the liver fell from 205% and 113% as assessed by detritiation activity to 78.9% and 29.8% respectively as assessed by solvent partition.

Both P and DOC gave acids when incubated with the hepatic and extra-hepatic tissues of both species. Higher yields of acids were obtained when DOC was the substrate, which was in agreement with a previous finding with rabbit liver [3].

Qualitative analysis of the acidic metabolites of [1,2-^{3}H]-deoxycorticosterone formed by the extrahepatic tissues of the rabbit and rat

Tritiated DOC (5 μCi) was incubated with the total low speed supernatants prepared from 10% and 25% homogenates of rat and rabbit tissues, as described in the Methods. The acidic fractions, isolated by solvent partition, were chromatographed on pre-coated silica gel plates (NEN, (OF)) in System 1. Figure 1 illustrates the radiochromatograms obtained with the rat and rabbit ovary and adrenal preparations. A clearly defined peak of radioactivity corresponding in mobility with the pregnenoic acid standard was defined in most of the radiochromatogram scans, with a broader more complex area of radioactivity being present in the area of the standard etienic acid. The chromatograms of the acids isolated from rat and rabbit liver were more complex than those from the

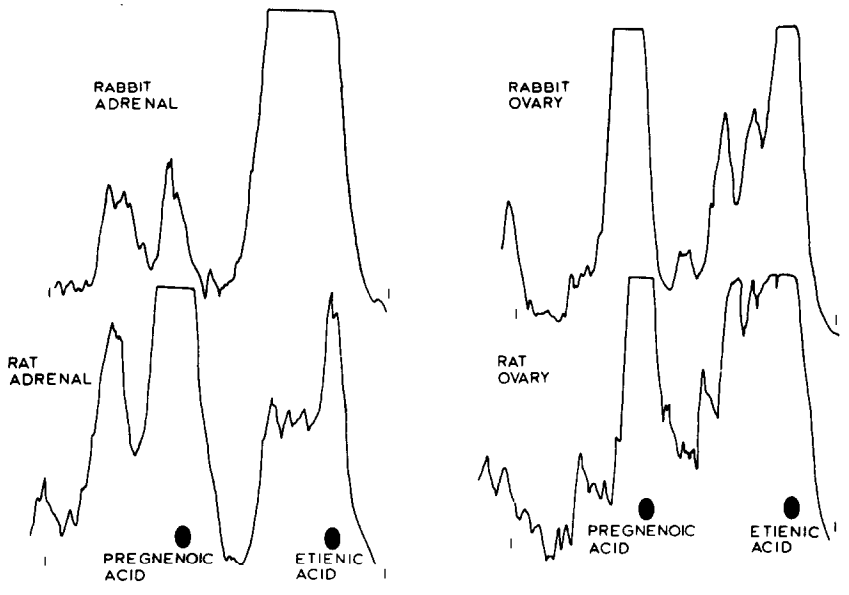


Fig. 1. Radiochromatogram scans of acidic metabolites of [1,2-^{3}H]-DOC isolated from rabbit and rat adrenal and ovary homogenates and chromatographed in System 1.

Table 3. Percentage formation of [^{3}H]-pregnenoic acid by tissue homogenate supernatants of the extrahepatic tissues of rat and rabbit

	Formation of acids		Isolated ^{3}H-pregnenoic acid			
	% of tritium* incubated		% of acids† isolated		% of tritium incubated	
Tissue	Rat	Rabbit	Rat	Rabbit	Rat	Rabbit
Adrenal	12.2	10.0	81.4	7.6	9.9	0.8
Ovary	1.7	4.4	25.8	56.9	0.4	2.5
Uterus	1.7	1.9	23.8	28.7	0.4	0.5
Kidney	1.0	2.2	39.6	23.5	0.4	0.5
Muscle	1.7	6.7	23.3	23.8	0.4	1.6

* 10% (w/v) Homogenates of adrenal and ovary; 25% (w/v) homogenates of the other tissues.
† Quantitated after chromatography of the free acids in System 1, methylation and chromatography in System 2.

extrahepatic tissues. This resulted from further metabolism of the acids by the hepatic homogenates to compounds which were not further investigated at the present time.

Identification of [1,2-^{3}*H*]-4-*pregnene*-3,20-*dione*-21-*oic acid as an extrahepatic tissue metabolite of* [1,2-^{3}*H*]-*deoxycorticosterone*

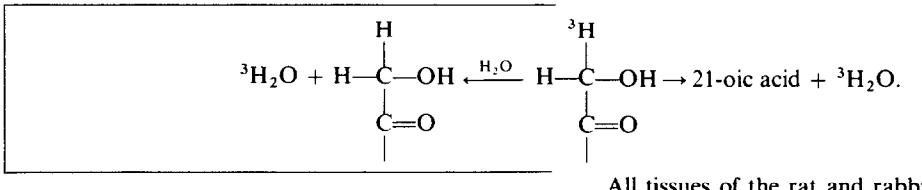
The peaks of radioactivity that co-chromatographed in t.l.c. System 1 with standard pregnenoic acid (R_F 0.35–0.37) were eluted and methylated, as previously described [5]. Chromatography of the methyl esters in System 2 gave a single peak of radioactivity in each case (R_F 0.30–0.45) that co-chromatographed with the authentic pregnoic acid methyl ester. Table 3 gives the recovery of acids and [^{3}H]-pregnenoic acid. Aliquots of the [^{3}H]-pregnenoic acids isolated from the rat adrenal and rabbit ovary incubates were taken as representative of the other tissues and were further characterised by crystallisation to constant specific activity with the authentic pregnenoic acid, according to the procedure previously described [6].

Detection of [1,2-^{3}*H*]-4-*androsten*-3-*one*-17*β*-*carboxylic acid*

Tritiated etienic acid was detected in the acidic fractions of rat and rabbit tissue homogenate incubates, but not quantitated. The broad zones of radioactivity that had the same mobility as the standard etienic acid when chromatographed in System 1 were further chromatographed in System 3, and gave complex radiochromatogram scans containing several radioactive peaks. One of these co-chromatographed with standard etienic acid R_F 0.50–0.53) and after methylation had the same mobility as the standard etienic acid methyl ester in System 2 (R_F 0.65–0.68).

DISCUSSION

The detritiation of the side-chain of 21-[^{3}H]-DOC has been shown to be linked to the formation of steroidal-21-oic acids by hamster liver [13] and involve the transfer of tritium to water [17]. Assay of this detriation activity has provided a convenient method for screening several tissues of the rat and rabbit for potential oxidation of the corticoid side-chain to carboxylic acids. However, when the detritiation activities were compared with the net yield of acids as assessed by solvent partition of the ring A tritiated metabolites, it was evident that the former procedure gave higher values of acid formation. One reason for this might be the occurrence of another enzyme catalysed reaction first noted by Monder and co-workers [15] with hamster liver. They also obtained lower net yields of acid than indicated by the detritiation experiments which they have attributed to an enzyme-catalysed enolisation of the side-chain involving the transfer of tritium to water but with the retention of the ketol side-chain. These reactions have been summarised [15] as follows:



All tissues of the rat and rabbit examined had significantly higher detritiation activity than the blank experiments, with the activity of the adrenals of both species being higher than the liver. Willingham and Monder [17] reported that the kidney and brain of the hamster had 53% and 29.4% of the detritiation activity of the liver, whereas the adrenal had only 7.9% the activity. All the extrahepatic tissues of the rabbit gave lower yields of acids than the liver. The adrenal and the ovary were of particular interest since they are sites of active steroid synthesis. Both were more active in acid formation than the non-endocrine tissue with the adrenal exhibiting up to 78.7% of the liver activity.

The extrahepatic tissues of the rat also gave lower yields of acids than the liver, which was more active than rabbit liver. The rat adrenal was not as active as rabbit adrenal when incubated at a 20,000:1 protein:substrate ratio (Table 2); however, it showed significantly higher activity when incubated with higher protein concentrations prepared from 10% homogenates (Table 3). These results suggest that both the liver and adrenals, and to a lesser extent, the ovaries, of the rat and rabbit may be important sites of steroid acid synthesis *in vivo*.

The identification of [^{3}H]-pregnenoic acid as a metabolite of [^{3}H]-DOC in the *in vitro* incubates of the extrahepatic tissues of the rat and rabbit is novel since it indicates that the oxidative metabolism of the corticoid side-chain is a widespread phenomenon in the living tissue of these two species. [^{3}H]-Pregnenoic acid has been identified as a hepatic metabolite of DOC in the rabbit, in a previous study [6] and with the hepatic tissues of several other species [9, 12, 13]. The *in vitro* yield of [^{3}H]-pregnenoic acid was very low with most of the extrahepatic tissues, except for rat adrenal, which gave a significantly higher yield of the acid than rabbit adrenal. It has been found, however, that following subcellular fractionation of rabbit adrenal, both the microsomal and mitochondrial fractions gave considerably higher yields of [^{3}H]-pregnenoic acid when incubated with [^{3}H]-DOC than with the unfractionated homogenate supernatant, which gave predominantly [^{3}H]-etienic acid [21]. This implies that a side-chain cleavage enzyme that gives rise to C-20-etienic acids may be present in the soluble fraction of adrenal homogenates, and might account for the low recovery of the 20-oxo-21-oic acid from the other extrahepatic tissues also. A significant proportion of the radiometabolites produced by the extrahepatic tissues were chromatographically more mobile than pregnenoic acid and probably corresponded to C-20-acids. [^{3}H]-Etienic acid was detected as one of the components of this fraction, and has been identified previously as a metabolite of DOC with rabbit liver [6] and with the adrenals and liver of several other species, as recently reviewed by Monder and Bradlow [15].

Though the metabolic significance of the formation of steroid carboxylic acids by extrahepatic tissues is uncertain, their formation by the endocrine glands of the rat and rabbit warrants further investigation.

Acknowledgement—This work was supported by a MRC Grant (MA-5403).

REFERENCES

1. Dey A. C. and Senciall I. R.: C-21- and 6-hydroxylation of progesterone by rabbit liver subcellular fraction. *Can. J. Biochem.* **55** (1977) 602–608.
2. Senciall I. R. and Dey A. C.: Acidic steroid metabolites: Evidence for the excretion of C-21-carboxylic acid metabolites of progesterone in rabbit urine. *J. steroid Biochem.* **7** (1976) 122–129.
3. Dey A. C. and Senciall I. R.: Acidic steroid metabolites: *In vitro* metabolism of tritiated progesterone and deoxycorticosterone by rabbit liver. *J. steroid Biochem.* **7** (1976) 167–170.
4. Senciall I. R., Harding C. A. and Dey A. C.: Acidic steroid metabolites: Species differences in the urinary excretion of acidic metabolites of progesterone. *J. Endocr.* **68** (1976) 169–170.
5. Senciall I. R., Harding C. A. and Dey A. C.: Acidic steroid metabolites: Predominant excretion of 20-oxopregnanoic acid metabolites of progesterone in rabbit urine. *J. steroid Biochem.* **9** (1978) 385–392.
6. Dey A. C. and Senciall I. R.: Acidic steroid metabolites: Biosynthesis of steroid carboxylic acids by rabbit liver microsomes and the involvement of a cytochrome P_{450}-independent mixed function oxidase system. *J. steroid Biochem.* **9** (1978) 1099–1104.
7. Senciall I. R.: Acidic steroid metabolites: Application of alumina adsorption chromatography in the differentiation of an acidic progesterone-^{14}C-metabolite fraction in rabbit bile and urine. *Biochem. Med.* **8** (1973) 423–431.
8. Allen J. G., Cooke A. M. and Thomas G. H.: The structure of some reaction products of the acidic urinary metabolites of progesterone. *J. Endocr.* **40** (1968) 153–163.
9. Schneider J. J.: *In vitro* conversion of deoxycorticosterone to some acidic metabolites. In *Proceed. Int. Cngr. Hormonal Steroids* (Edited by L. Martini and A. Pecile). Academic Press, New York (1964) Vol. 1, pp. 127–135.
10. Bradlow H. L., Zumoff B., Monder C., Lee H. J. and Hellman L.: Isolation and identification of four new carboxylic acid metabolites of cortisol in man. *J. clin. Endocr. Metab.* **37** (1973) 811–818.
11. Senciall I. R., Riley C., Rahal S., Dey A. C., Harding C. A. and Acharya M.: Effect of structure on the excretion of steroidal acids in human urine and their potential colorimetric assay as 17-oxogenic steroids. *Clin. Biochem.* **10** (1977) 32–37.
12. Martin K. O. and Monder C.: Oxidation of corticosteroids to steroidal-21-oic acids by human liver enzyme. *Biochem.* **15**, (1976) 576–582.
13. Lee H. J. and Monder C.: Oxidation of corticosteroids to steroidal carboxylic acids by an enzyme preparation from hamster liver. *Biochem.* **16** (1977) 3810–3814.
14. Monder C. and Wang P. T.: Oxidation of 21-dehydrocorticosteroids to steroidal 20-oxo-21-oic acids by ketoaldehyde dehydrogenase of sheep liver. *J. steroid Biochem.* **4** (1973) 153–162.
15. Monder C. and Bradlow H. L.: Carboxylic acid metabolites of steroids. *J. steroid biochem.* **8** (1977) 897–908.
16. Monder C. and Wang P. T.: Oxidation of 21-dehydrocorticosteroids to steroidal-21-oic acids by an aldehyde dehydrogenase of sheep adrenal. *J. biol. Chem.* **248** (1973) 8547–8554.
17. Willingham A. K. and Monder C.: Evidence for the metabolic lability of the C-21-hydrogens of corticosteroids. *Endocr. Res. Commun.* **1** (1974) 145–153.
18. Oh S. W. and Monder C.: Synthesis of corticosteroids derivatives containing the 20β-ol-21-al side-chain. *J. org. Chem.* **41**, (1976) 2477–2480.
19. Willingham A. K. and Monder C.: Synthesis of 21-[^{3}H]-corticosteroids. *Steroids* **22** (1973) 539–546.
20. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with Folin-Phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
21. Senciall I. R. Unpublished observations.