ACTA CHEMICA SCANDINAVICA 27 (1973) 1240–1248

Transformation of Steroids by Cell-free Preparations of *Penicillium lilacinum* NRRL 895
II. Hydrolysis of Steroid Esters

KJELL CARLSTRÖM and KARL KROOK

Division of Applied Microbiology, Royal Institute of Technology and the Department of Obstetrics and Gynaecology, Sabbatsberg Hospital, Karolinska Institutet, Stockholm, Sweden*

Supernatants obtained after centrifugation at 100 000 g of homogenates of *Penicillium lilacinum* NRRL 895 were shown to contain inducible steroid esterase activity. Acetoxyl groups in positions 3α, 3β, 3(phenolic)17α(*sec*), 17β(*sec*), 20α, and 21 were hydrolyzed to the corresponding alcohols. The 6β, 11α, 17α(*tert*) and 20β-acetates were resistant to hydrolysis. Testosterone propionate was partially hydrolyzed, whereas testosterone oenanthate, benzoate, and hemisuccinate were unaffected. The influence of pH, of metals and of inhibitors was studied.

Hydrolysis of steroid esters is brought about by several organisms and was one of the first microbial steroid transformations to be reported.[1] Few systematic studies have, however, been carried out in cell-free systems. Partially purified steroid esterases from *Nocardia restrictus* and *Cylindrocarpon radicicola* have been studied by Sih and co-workers.[2–4] They found interesting differences in substrate specificity and stability properties of these two enzymes. Steroid lactonase and esterase activities in cell-free preparations of *Cephalosporium acremonium* were investigated by Holmlund and Blank, who claimed two different enzymes as being responsible for these activities.[5] They also prepared cellfree extracts of *Streptomyces roseochromogenes* and *Flavobacterium dehydrogenans var. hydrolyticus*, having steroid inducible esterase activity. However they did not pursue their study of the steroid esterase activity in these organisms. More recently, Lestrovaya *et al.* described the preparation of cell-free extracts from *Mycobacterium album*, containing inducible steroid esterase activity.[6,7]

The steroid transforming capacity of the fungus *Penicillium lilacinum* is well documented.[1] Hydrolysis of testololactone to testolic acid has been demonstrated by Prairie and Talalay in soluble and partially purified enzyme

* Present address.

preparations from this microbe.[8] During a study of the side chain cleavage of some progesterone derivatives in cell-free preparations of the fungus at this laboratory, it was observed that the 21-acetoxyl group of desoxycorticosterone acetate was easily hydrolyzed. It is well known that the hydrolysis of steroid esters and lactones will play an important role in the breakdown of steroids by several microorganisms.[1] This has led us to investigate the steroid esterase activity of *P. lilacinum* in somewhat greater detail.

Abbreviations and trivial names. DFP: diisopropyl fluorophosphate; GLC: gas liquid chromatography; GC-MS: gas chromatography-mass spectrometry; t_R: retention time relative to 5α-cholestane; Silyl: trimethylsilyl; TLC: thin layer chromatography; UV: ultraviolet. Androsterone: 3α-hydroxy-5α-androstane-17-one; Desoxycorticosterone: 21-hydroxy-4-pregnene-3,20-dione; Dehydroepiandrosterone: 3β-hydroxy-5-androsten-17-one; Epiandrosterone: 3β-hydroxy-5α-androstane-17-one; Epitestosterone: 17α-hydroxy-4-androsten-3-one; Oestrone: 3-hydroxy-1,3,5(10)-oestratriene-17-one; Progesterone: 4-pregnene-3,20-dione; Testololactone: 17a-oxa-4-androsten-3,17-dione; Testolic acid: 13-hydroxy-3-oxo-13,17-*seco*-4-androsten-17-oic acid; Testosterone: 17β-hydroxy-4-androsten-3-one.

MATERIALS AND METHODS

Steroids. All steroids were checked for purity by GLC and/or TLC. Steroid ester substrate solutions in ethanol were prepared immediately before use.

Androsterone and its acetate, 5α-cholestane, desoxycorticosterone acetate, dehydroepiandrosterone and its acetate, epiandrosterone and its acetate, oestrone, progesterone, 11α-hydroxyprogesterone and its acetate, 17α-hydroxyprogesterone, testosterone and its acetate, benzoate, oeanthate, and propionate were obtained from Sigma Chemical Company, St. Louis, Mo. Desoxycorticosterone, 20α- and β-hydroxy-4-pregnene-3-one were obtained from Ikapharm Ltd., Ramat-Gan, Israel. Epitestosterone and its acetate, 20α- and β-acetoxy-4-pregnene-3-one, oestrone acetate, 6β-hydroxyprogesterone, 17α-acetoxyprogesterone and testosterone hemisuccinate were obtained from Steraloids Inc., Pawling, N.Y.

6β-Acetoxy progesterone was prepared from the corresponding alcohol by acetylation with acetic anhydride in pyridine. The homogeneity of the acetylation product was checked by TLC and by hydrolysis to the parent alcohol.

Other chemicals. Solvents were of reagent grade and with the exception of the ethanol they were redistilled before use. The water used in the enzyme experiments was deionized and distilled in an all glass apparatus. All other chemicals were of reagent grade and were used without further purification. DFP was obtained from the Research Institute of National Defence (FOA), Ursvik, Sweden. Sodium phosphate buffer 0.06 M pH 7.2 was used throughout the investigation except in the stability test and occasionally in the washing of the cells where 0.06 M Tris-HCl pH 7.2 was used. The type of buffer does not affect the enzyme activity or the stability in frozen state.

Growth of organism and preparations of cell-free extracts. P. lilacinum NRRL 895 was grown on Czapek-Dox medium, induced, washed and frozen as described previously.[9] Testosterone acetate was used as inducer (70 mg in 2 ml of dimethylformamide per 300 ml culture). Cell-free extracts were prepared by grinding with sand as described previously but before the final centrifugation at 100 000 *g* the homogenates were frozen at −22° for at least 24 h. Before the 100 000 *g* centrifugation they were thawed and centrifuged at 6000 rpm for 15 min. A considerable amount of inactive precipitate is removed in this way. The supernatant was centrifuged in a Beckman-Spinco Model L ultracentrifuge at 100 000 *g* for 60 min. Phosphate buffer was added to the supernatant to give a fourfold dilution of the original homogenate. This diluted extract was immediately used in the enzyme experiments.

Transformation of steroid substrate. To 1 ml of diluted 100 000 *g* supernatant in a centrifuge tube, 0.3 μmol of steroid ester substrate in 10 μl of ethanol was added. After

Table 1. t_R- and R_F-values for the TLC and GLC systems described in the text.

Compound	t_R, OV-17 (1,2,3,4)	t_R, XE-60 (5)	t_R, SE-30 (6)	Internal standard in quantitative GLC	R_F-value
Cholestane	1.00	1.00	1.00		
Oestrone	1.47 (1)		0.51	Cholestane	0.52
Oestrone acetate	1.76 (1)			»	0.58
Testosterone	1.39 (2)			»	0.20
Testosterone acetate	1.70 (2)			»	0.47
Testosterone propionate	2.23 (2)			»	0.48
Testosterone oenanthate					0.55
Testosterone benzoate					0.54
Testosterone hemisuccinate					0.00
Testosterone silyl ether	0.95 (4)	2.17	0.67		
Epitestosterone	1.36 (1)			Cholestane	0.21
Epitestosterone acetate	1.61 (1)			»	0.44
Epitestosterone silyl ether	0.81 (4)	1.74	0.60		
Androsterone	0.87 (1)			Progesterone	0.26
Androsterone acetate	1.05 (1)			»	0.63
Androsterone silyl ether		0.87	0.46		
Epiandrosterone	0.93 (1)			Progesterone	0.27
Epiandrosterone acetate	1.24 (1)			»	0.66
Epiandrosterone silyl ether		1.27	0.56		
Dehydroepiandrosterone	0.89 (1)			Progesterone	0.24
Dehydroepiandrosterone acetate	1.18 (1)			»	0.58
Dehydroepiandrosterone silyl ether		1.20	0.51		
20α-Hydroxy-4-pregnene-3-one	2.22 (2)			Cholestane	0.23
20α-Acetoxy-4-pregnene-3-one	2.79 (2)			»	0.46
20α-Trimethylsilyloxy-4-pregnene-3-one	1.78 (4)	4.10	1.21		
20β-Hydroxy-4-pregnene-3-one	2.00 (2)			Cholestane	0.25
20β-Acetoxy-4-pregnene-3-one	2.63 (2)			»	0.48
Progesterone	2.15 (1)				
6β-Hydroxyprogesterone					0.17
6β-Acetoxyprogesterone					0.41
11α-Hydroxyprogesterone					0.05
11α-Acetoxyprogesterone					0.25
17α-Hydroxyprogesterone	3.27 (3)			Cholestane	0.31
17α-Acetoxyprogesterone	4.60, 2.27, 2.00 (3)			»	0.34
Desoxycorticosterone					0.20
Desoxycorticosterone acetate					0.40
Desoxycorticosterone silyl ether			1.87		

Indications	Column temp.	Carrier gas inlet pressure	Retention time for cholestane, min
(1)	237°	2.7 kp/cm²	16.2
(2)	247°	2.7 kp/cm²	11.5
(3)	257°	2.7 kp/cm²	8.2
(4)	250°	2.7 kp/cm²	9.8
(5)	228°	2.4 kp/cm²	7.35
(6)	240°	Gas flow 35 ml/min	8.35

mixing on a Vortex mixer for 5 sec, incubation took place on a shaking table at +26°. All incubations were run as duplicates. After the desired incubation time, the reaction was terminated in two ways, depending upon the type of steroid ester substrate. For esters of C_{18} and C_{19} steroids, 0.1 ml of 1 M HCl was added and the steroids were extracted with 4 ml of chloroform. The chloroform layer was dried over Na_2SO_4 and subjected to analysis. For esters of C_{21} steroids, 4 ml of acetone was added and the tube was shaken thoroughly. After cooling under running tap water for 30 min, the precipitate was removed by centrifugation and the supernatant was subjected to analysis. This latter procedure was used because of the water solubility of certain hydroxylated progesterone derivatives, *e.g.* desoxycorticosterone. Blanks without steroid substrate added were prepared and treated similarly. At the dilution of the extract used in these incubations, no interfering compounds from the inducer or the fungus could be detected. Unless otherwise stated, this procedure was used throughout the study and is referred to as the standard procedure.

Assay techniques. Before quantitative analysis, the samples were subjected to a qualitative TLC on Silica gel GF_{254} plates (100 × 200 mm) with ethyl acetate:benzene:hexane 5:4:4 as solvent (single run). Δ^4-3-ketosteroids were visualized in 254 nm UV light, other steroids with 10 % $SbCl_5$ in chloroform. In the alcohol/ester pairs oestrone/oestrone acetate and 17α-hydroxyprogesterone/17α-acetoxyprogesterone, the ester and the alcohol were poorly separated, and in these cases qualitative GLC on OV-17 was performed. The R_F-values in the TLC system are given in Table 1.

Samples showing formation of hydroxysteroids were quantitatively analyzed by GLC. A Perkin-Elmer F-11 MK II gas chromatograph with flame ionization detection was used. The column was a 2 m × 3 mm i.d. glass column, packed with 2.5 % OV-17 on AW-DMCS Chromosorb W, 80-100 mesh. t_R values are given in Table 1. The mean recovery in the whole analytical procedure, including the chloroform extraction was ≥ 95 % for testosterone and ≥ 95 % for testosterone acetate. The variation between the individual samples in the duplicate incubations for testosterone and testosterone acetate is given in Table 2.

Desoxycorticosterone and its acetate were quantitated by measurement of absorbance at 240 nm in a Beckman DB UV-VIS spectrophotometer. The ester and the alcohol were separated by TLC before the quantitation.

Protein assays were made by the biuret method.[10]

Identifications. Hydroxy steroids formed by enzymatic hydrolysis were identified on the basis of the following criteria:

(1) R_F-values in the TLC.

(2) t_R as free alcohols on 2.5 % OV-17, and as silyl ethers on 2.5 % XE-60 and on 1.5 % SE-30. Prior to the formation of the silyl esters, the hydroxysteroids were isolated by TLC.

(3) GC-MS analysis of the silyl ether derivatives in an LKB 9000 gas chromatograph-mass spectrometer. The conditions were the same as those previously described, except that the column temperature was 240° and the spectra were recorded in the *m/e* range 0 – 800.[9]

Table 2. Variation in duplicate incubations for testosterone and testosterone acetate. Single analysis of each sample. N = number of duplicate incubations. S.D. = $\sqrt{\sum d^2/2N}$.

Expressed in μmol steroid per tube	μmol steroid/tube	S.D.	N
Testosterone	0.157 (0.028 – 0.246)	±0.017	10
Testosterone acetate	0.182 (0.041 – 0.281)	±0.014	13
Expressed in mol % steroid	**Mol % steroid**	**S.D.**	**N**
Testosterone	62.3 (9.6 – 85.4)	±2.3	16
Testosterone acetate	37.7 (14.6 – 90.4)	±2.3	16

RESULTS

Effect of different steps in the preparation of the cell free extracts. The treatment of the culture with inducer steroids is an essential step for obtaining esterase active cell free extracts. To one of two 100 ml aliquots from the same 6 day culture, 35 mg of testosterone acetate in 1 ml dimethylformamide was added and to the other aliquot the dimethylformamide only. After 14 h of incubation, cell-free extracts were prepared and incubated with testosterone acetate for 15 min according to the standard procedure. With the extract from the steroid treated cells, 70.0 % of the substrate was hydrolyzed whereas the non induced cells yielded a completely inactive extract. Progesterone as well as testosterone acetate may be used as inducer.

Grinding the cells with sand yielded the most active cell free extracts and proved far more effective than treatment of the cells in an Elvehjem-Potter homogenizer or by sonication.

The crude homogenate obtained after the first 6000 rpm centrifugation is fairly stable at $-22°$, losing about 50 % of its activity in the course of three weeks. The stability of this preparation at three different temperatures is given in Fig. 1.

The freezing and thawing procedure removes a considerable amount of inactive protein. In one experiment the specific activity, expressed in μmoles testosterone acetate hydrolyzed by 1 mg protein in 15 min, increased from 0.78 to 1.24. The precipitate contained no esterase activity. This has previously been observed by Rahim and Sih for the steroid esterase of *C. radicicola*.[4] Attempts to purify the extracts by treatment with freeze cold CH_2Cl_2 according to Sih *et al.*[2] proved unsuccessful, despite extensive purification of the solvent. The CH_2Cl_2 treatment caused a 95 % inhibition of the activity.

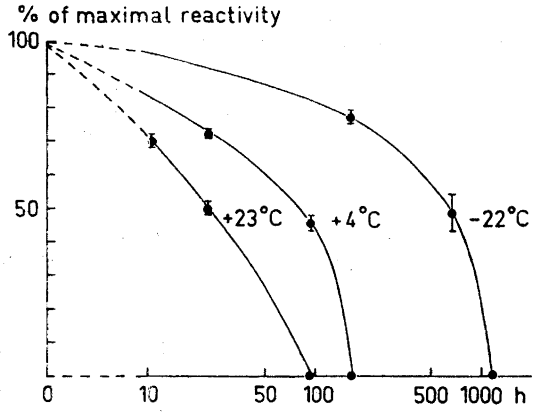

Fig. 1. Stability of crude cell free esterase preparations of *P. lilacinum* at three different temperatures.

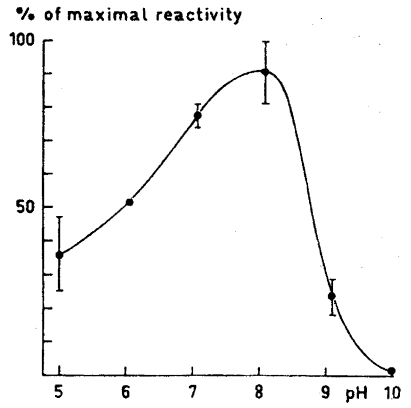

Fig. 2. pH-activity relationship for the hydrolysis of testosterone acetate by 100 000 *g* cell-free supernatants of *P. lilacinum*. Incubation time 2 min. Maximal hydrolysis of testosterone acetate = 15.9 %.

Enzymatic nature of the reaction. Influence of pH. Boiled extracts (100° for 5 min) were completely inactive, thereby demonstrating the enzymatic nature of the reaction. Hydrolysis of testosterone acetate was optimal at pH 8.1 (Fig. 2). The pH profile is similar to that obtained by Sih and co-workers for the steroid esterase of *N. restrictus*.[3]

Effect of inhibitors. Aliquots (1.0 ml) of the cell-free extract were preincubated with enzyme inhibitors at 1×10^{-3} M concentration for 15 min at 26°, The inhibitors were added in 0.1 ml phosphate buffer solution. Incubation with testosterone acetate was subsequently carried out for 2 min according to the standard procedure. In the controls without inhibitors 18.0 % of the substrate was hydrolyzed. $HgCl_2$ caused an inhibition of 100 %, and *p*-hydroxymercuri benzoate and NaF of 89 and 65 %, respectively. KCN, EDTA, 8-hydroxyquinoline, and NaN_3 were without significant effect.

The inhibition caused by the organophosphorus compound DFP is strongly dependent upon the concentrations of enzyme and inhibitor and of the preincubation time. The DFP was added as a freshly prepared 10 % (v/v) solution in ethanol. To the controls only ethanol was added. The results are given in Table 3. In concentrated extracts high concentrations of DFP are necessary for obtaining inhibition.

Table 3. Effect of DFP on the hydrolysis of testosterone acetate by 100 000 *g* cell-free supernatants from *P. lilacinum*. Preparation 1 is diluted with buffer according to the standard procedure, preparations 2 and 3 are undiluted.

Preparation	Protein mg/ml	DFP added	Preincubation time, min.	% hydrolysis	% inhibition
1	0.120	None		76.7	–
1	0.120	1×10^{-3}M	20	70.3	8.3
1	0.120	1×10^{-3}M	40	53.9	29.8
1	0.120	1×10^{-2}M	40	0	100
2	0.520	None		85.4	–
2	0.520	1×10^{-2}M	20	81.5	4.6
2	0.520	1×10^{-2}M	40	31.2	63.5
3	1.370	None		100	–
3	1.370	1×10^{-2}M	30	100	–

Effect of metal ions. 1×10^{-3} M concentrations of $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CuCl_2$, $NiCl_2$, and $CoCl_2$ had no significant effect upon the enzyme activity (incubation with testosterone acetate for 15 min according to the standard procedure, in the controls 40.4 % of the substrate was hydrolyzed). The type of buffer medium did not influence the effect of metals.

Substrate specificity. Different steroid esters were incubated with the enzyme preparation for 15 min following the standard procedure. The incubations were run in two series: Testosterone acetate plus esters of C_{18} and C_{19} steroids, and testosterone acetate plus esters of C_{21} steroids. In the two series,

Table 4. Substrate specificity of the steroid esterase activity in 100 000 *g* cell-free supernatants from *P. lilacinum*.

Steroid ester substrate	Position	% relative hydrolysis (testosterone acetate = 100 %)
Testosterone acetate	17β(sec)	100.0
Testosterone propionate	17β(sec)	16.9
Testosterone oenanthate	17β(sec)	0
Testosterone benzoate	17β(sec)	0
Testosterone hemisuccinate	17β(sec)	0
Epitestosterone acetate	17α(sec)	96.3
Androsterone acetate	3α	28.8
Epiandrosterone acetate	3β	92.8
Dehydroepiandrosterone acetate	3β	99.3
Oestrone acetate	3 (phenolic)	98.8
20α-Acetoxy-4-pregnene-3-one	20α	86.5
20β-Acetoxy-4-pregnene-3-one	20β	0
6β-Acetoxyprogesterone	6β	0
11α-Acetoxyprogesterone	11α	0
17α-Acetoxyprogesterone	17α (tert)	0
Desoxycorticosterone acetate	21	85.0

testosterone acetate was hydrolyzed to 80.3 % and 83.8 %, respectively. The hydroxy steroids formed were identified as described in Identifications. In all cases, TLC, GLC and mass-spectrometric properties were identical to those of the authentic reference compounds. The results are given in Table 4. A check was made to ensure that incubation with buffer only did not cause any hydrolysis of the steroid esters studied.

Testosterone acetate was readily hydrolyzed and testosterone propionate to a lesser degree. Testosterone oenanthate, benzoate, and hemisuccinate were unaffected. Acetoxyl groups at position 3α, 3β, 3 (phenolic), 17α (sec), 17β (sec), 20α and 21 were hydrolyzed whereas 6β, 11α, 17α (tert) and 20 β-acetates were resistant to hydrolysis.

DISCUSSION

Most of the properties shown by the steroid esterase activity of *P. lilacinum* resemble those reported for other microbial enzymes of this kind.[2–7] However when making such comparisons, the state of purification of the different esterase preparations must be taken into consideration. It is well known that such preparations usually contain more than one esterase and that substrate specificity as well as sensitivity to inhibitors may vary considerably for the individual esterases in one preparation.

A system for the classification of esterases into A-, B-, and C esterases on the basis of their sensitivity to organophosphorus inhibitors was introduced by Aldridge.[11] The steroid esterase activity in *P. lilacinum* is relatively insensitive to DFP but a definite classification is not possible with the crude preparation used. The insensitivity might be due to real insensitivity of the esterase or

to a rapid hydrolysis of the DFP by the esterase itself or by more specific DFP-hydrolyzing enzymes ("DFP-ase, Sarinase"). This might also explain the relative inertness towards organophosphorus inhibitors shown by the steroid esterase activity in *Septomyxa affinis* and of acetyl esterase preparations of fungal and plant origin.[12-14] DFP-hydrolyzing enzymes have previously been demonstrated in microorganisms.[15]

Like other microbial steroid esterases, this enzyme is sensitive to SH-reacting inhibitors such as *p*-hydroxymercuri benzoate.[2-5] However, during the preliminary experiments it was difficult to obtain reproducible results with this inhibitor for different cell-free preparations. The degree of inhibition reported for other enzymes of this kind also varies considerably.[2-5] It has been shown for the acetyl esterase in wheat germ that the inhibition caused by SH-reacting inhibitors strongly depends upon the type of substrate used and it was concluded that a typical SH-enzyme nature of this enzyme seemed doubtful.[13] This might also be valid for the microbial steroid esterases. With the exception of Hg^{2+}, metal ions and metal complexing and chelating agents had no significant influence upon the esterase activity. Fluoride exerted a strong inhibitory effect (65 % at 1×10^{-3} M) and the degree of inhibition was not dependent upon the type of buffer used (phosphate or Tris-HCl). The inhibition caused by fluoride is probably not due to any interaction with metals. It is well known that liver esterase and other hydrolytic enzymes not depending upon metals are strongly inhibited by fluoride.[16]

The substrate specificity roughly resembles that reported for the steroid esterases in *N. restrictus* and *C. radicicola*.[2-4] The differences shown might be of quantitative rather than qualitative character. As expected, all three preparations failed to hydrolyze the tertiary acetoxyl group in 17α-acetoxyprogesterone. However, a few cases of microbial hydrolysis of tertiary 17α-acetyl esters have been reported.[1,17] Of the epimeric 20-hydroxy-4-pregnene-3-ones, the 20α-epimer was easily hydrolyzed whereas the 20β-epimer resisted enzymatic hydrolysis. The inertness of the 20β-ester might be explained by steric hindrance from the C_{18} and C_{21} methyl groups and from the 12α-hydrogen. Similar selectivity in the hydrolysis of epimeric 20-acetates has previously been demonstrated in *Aspergillus ochracaeus*.[18] Hydrolysis of 20β-acetates has, however, been reported for *Flavobacterium dehydrogenans* and for the steroid esterase from *N. restrictus*.[1,2]

Acknowledgement. The GC-MS analysis was carried out at the Department of Chemistry 1, Karolinska Institutet, Stockholm, Sweden.

REFERENCES

1. Charney, W. and Herzog, H. L. *Microbial Transformations of Steroids. A Handbook*, Academic, New York and London 1967.
2. Sih, C. J., Laval, J. and Rahim, M. A. *J. Biol. Chem.* **238** (1963) 566.
3. Rahim, M. A. and Sih, C. J. *J. Biol. Chem.* **241** (1966) 3615.
4. Rahim, M. A. and Sih, C. J. *Microbial Steroid Esterases.* In Colowick, S. P. and Kaplan, N. O., Eds., *Methods of Enzymology*, Academic, New York and London 1969, Vol. 15, p. 675.
5. Holmlund, C. E. and Blank, R. H. *Arch. Biochem. Biophys.* **109** (1965) 29.

6. Lestrovaya, N. N., Koshchenko, K. A. and Ksandopulo, G. B. *Izv. Akad. Nauk SSSR, Ser. Biol.* **5** (1969) 773; *Chem. Abstr.* **72** (1970) 62907.
7. Lestrovaya, N. N., Ksandopulo, G. B. and Karasevich, Zh. G. *Mikrobiologiya* **40** (1971) 461; Chem. Abstr. **75** (1971) 85547.
8. Prairie, R. L. and Talalay, P. *Biochemistry* **2** (1963) 203.
9. Carlström, K. *Acta Chem. Scand.* **26** (1972) 1718.
10. Lowry, O. H., Rosebrough, N. J., Farr. A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
11. Aldridge, W. N. *Biochem. J.* **53** (1953) 110.
12. Miller, T. L. *Biochim. Biophys. Acta* **270** (1972) 167.
13. Oosterbaan, R. A. and Jansz, H. S. *Cholinesterases, Esterases and Lipases (Review)*. In Florkin, M. and Stotz, E. H., Eds., *Comprehensive Biochemistry*, Elsevier, Amsterdam, London and New York 1965, Vol. 16.
14. Krisch, K. *Carboxylic Ester Hydrolases (Review)*. In Boyer, P. D., Ed., *The Enzymes*, 3rd Ed. Academic, New York and London 1971, Vol. V, p. 43.
15. Mounter, L. A., Baxter, R. F. and Chanutin, A. *J. Biol. Chem.* **215** (1955) 699.
16. Wiseman, A. *Effect of Inorganic Fluoride on Enzymes (Review)*. In Smith, F. A., Ed., *Handbuch der experimentellen Pharmakologie*, Springer-Verlag, Berlin, Heidelberg and New York 1970, p. 48.
17. Noguchi, S., Otsuka, H. and Takahashi, T. *Japan. Pat.* 71 16. 149, May 1, 1971; Chem. Abstr. **75** (1971) 74910.
18. Lewbart, M. L. and Schneider, J. J. *J. Org. Chem.* **29** (1964) 2559.

Received November 7, 1972.