DECOMPOSITION OF CELLULOSE BY THE FUNGUS CURVULARIA LUNATA WAKKER

II—Factors affecting the elaboration of cellulolytic enzymes

by

G. M. Verma, D. D. Sahgal, R. K. Verma, T. S. A. Padmanabhan and P. K. Vijayaraghavan

Defence Research Laboratory (Stores), Kanpur

ABSTRACT

A study of the effect of nutritional factors on the elaboration of extracellular cellulolytic enzyme by the fungus *Curvularia lunata* has been carried out. A modified Omeliansky's medium has been formulated substituting ammonium sulphate by ammonium chloride in the Omeliansky's medium and incorporating sodium carboxy methyl cellulose as cellulosic substrate. Use of indole-acetic acid in the growth medium has been found to increase the *in vivo* as well as *in vitro* cellulolytic activity. The fungus when grown in the presence of subthreshold concentrations (i.e. at 0·001%) of sodium pentachlorophenate elaborated an extracellular cellulolytic enzyme of enhanced activity.

Introduction

In the earlier paper[1] from this laboratory investigations carried out on the cellulolytic enzyme elaborated by the fungus *Curvularia lunata* Wakker, have been reported. The present paper deals with the study of the nutritional requirements of the fungus *C.lunata* with respect to its capacity to elaborate an active extracellular cellulolytic enzyme. Nutritional requirements for the growth of the fungus have been reported in the literature[2] but these studies do not throw any light on the cellulose decomposing capacity of this fungus *in vitro*. A culture medium from which the fungus will elaborate an active extracellular enzyme may not necessarily give maximum growth of the fungus as growth is the sum total of many enzyme systems working *in vivo* within the organism. Studies were, therefore, undertaken to formulate a culture medium which may or may not produce optimum growth of the fungus but which will enable the fungus to elaborate an active cellulolytic enzyme.

Experimental Procedure*

Media—The composition of the different media tried are given below:

1. *Omeliansky's medium*[3]

Ammonium sulphate $[(NH_4)_2 SO_4]$	1·0 gm
Dipotassium hydrogen phosphate (K_2HPO_4)	1·0 gm

* The chemicals used in these investigations were of A.R. quality.

Magnesium sulphate ($MgSO_4 \cdot 7\ H_2O$)	0·5 gm
Calcium carbonate ($CaCO_3$)	2·0 gms
Sodium chloride (NaCl)	Traces
Cellulosic substrate (cuprammonium cellulose)	20·0 gms
Distilled water	1000 ml

pH 6·2—6·4

2. *Greathouse's medium*[4]

Dipotassium hydrogen phosphate (K_2HPO_4)	1·40 gms
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0·74 gm
Ammonium nitrate ($NH_4\ NO_3$)	1·00 gm
Calcium carbonate ($CaCO_3$)	5 mgms
Sodium chloride (NaCl)	5 mgms
Ferrous sulphate ($FeSO_4$)	1 mgm
Zinc sulphate ($ZnSO_4$)	1 mgm
Manganese sulphate ($MnSO_4$)	1 mgm
Cellulosic substrate (cuprammonium cellulose)	20·0 gms
Distilled water	1000 ml

pH 6·2—6 4

3. *Mandel's medium*[5]

Ammonium nitrate ($NH_4\ NO_3$)	3·00 gms
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	2·22 gms
Potassium dihydrogen phosphate ($KH_2\ PO_4$)	2·59 gms
Dipotassium hydrogen phosphate (K_2HPO_4)	2·21 gms
Cellulosic substrate (cuprammonium cellulose)	20·0 gms
Distilled water	1000 ml

pH 6·5

4. *Martin's medium*[6]

Ammonium nitrate ($NH_4\ NO_3$)	3·0 gms
Potassium dihydrogen phosphate ($KH_2\ PO_4$)	0·25 gm
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0·25 gm
Potassium chloride (KCl)	0·25 gm
Cellulosic substrate (cuprammonium cellulose)	20·0 gms
Distilled water	1000 ml

pH 6·4

5. *Fries medium*[7]

Ammonium tartarate [$C_4H_4O_6\ (NH_4)_2$]	5·0 gms
Ammonium nitrate (NH_4NO_3)	1·0 gm
Potassium dihydrogen phosphate (KH_2PO_4)	1·0 gm
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0·5 gm

Sodium chloride (NaCl)	0·1 gm
Calcium chloride ($CaCl_2$)	0·1 gm
Boric acid (H_3BO_3)	0·06 gm
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0·25 gm
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0·55 gm
Zinc sulphate ($ZnSO_4$)	5 mgms
Molybdic acid ($H_2 MoO_4$)	0·02 mgm
Cellulosic substrate (cuprammonium cellulose)	20·0 gms
Distilled water	1000 ml

pH 6·4—6·8

6. *Czapek-Dox's medium*[8]

Sodium nitrate ($NaNO_3$)	3·0 gms
Potassium dihydrogen phosphate (KH_2PO_4)	1·0 gm
Potassium chloride (KCl)	0·5 gm
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0·5 gm
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	traces
Zinc sulphate ($ZnSO_4$)	traces
Cellulosic substrate (cuprammonium cellulose)	20·0 gms
Distilled water	1000 ml

pH 4·2—4·4

Methods—In general the procedures for preparation of growth medium, inoculation of fungus, separation of the extracellular enzyme and estimation of enzymatic activity were essentially the same as described earlier[1]. Reducing sugars formed as a result of enzyme action were estimated by the method of Shaffer and Somogyi[9]. For assessment of toxic effects of sodium pentachlorophenate, the radial growth of the fungus was measured on solidified agar medium in Petri dishes 15 cms. in diameter. The constituents of the agar medium were the same as in Omeliansky's medium[3] (described earlier) except that (*a*) ammonium sulphate was replaced by ammonium chloride and (*b*) cuprammonium cellulose was replaced by sodium salt of carboxymethyl cellulose*. The fungicide was used in different concentrations ranging from ·0001 to 0·02%.

Results

Effect of carbohydrate source on elaboration of enzyme—Preliminary investigations† carried out in this laboratory showed that the growth of the fungus on media containing different sugars (in the absence of cellulose) was satisfactory. To find out the effect of sucrose on the elaboration of the enzyme, the fungus was grown on Czapek-Dox medium[8] containing (*a*) mineral solution only (*b*) 2% sucrose+2% cellulose (Whatman filter paper) as sources of carbohydrate and (*c*) 2% cellulose (Whatman filter paper) only as a source of carbohydrate.

* D.S. 0·45 to 0·55.

† Unpublished work DRL (S) Kanpur.

The activity of cellulolytic enzyme in the metabolic liquor was assessed after 28 days of growth. The results are given in Table I below:—

TABLE I

Effect of carbohydrate source on elaboration of enzyme

Media	Set No.	Cellulase activity (% hydrolysis of cellulose)
Czapek-Dox's medium (no carbon source)	1	Nil
	2	Nil
Czapek-Dox's medium+filter paper+sucrose	1	Nil
	2	Nil
Czapek-Dox's medium + filter paper	1	8·8
	2	13·8

The data in Table I above show that the elaboration of cellulolytic enzyme does not take place when the carbohydrate source is (*a*) sucrose alone and (*b*) sucrose+filter paper. Cellulolytic enzymes are elaborated only when cellulose is the sole source of carbohydrate. The presence of the simpler carbohydrate i.e. sucrose possibly checks the elaboration of the enzyme.

Elaboration of the cellulolytic enzyme in different media—The fungus was grown on five different media *viz.* Omeliansky[3], Greathouse[4], Mandel[5], Martin[6] and Fries[7] using cuprammonium cellulose as source of carbohydrate. In each case the enzymatic activity of the metabolic liquor was assessed at different periods of growth of the fungus. The results are summarised in Table II below:—

TABLE II

Elaboration of cellulolytic enzyme in different media

No.	Media	Cellulase activity (% hydrolysis of cellulose)			
		Period of growth of fungus			
		7 days	14 days	21 days	28 days
1	Omeliansky's medium	Nil	Nil	7·0	9·0
2	Greathouse's medium	1·4	2·5	Nil	8·0
3	Mandel's medium	Nil	Nil	Nil	Nil
4	Martin's medium	7·0	Nil	Nil	Nil
5	Fries' medium	3·0	7·0	Nil	9·0

It is evident from Table II above that the fungus elaborates a more active cellulolytic enzyme in Omeliansky's and Fries' media as compared to Greathouse's, Mandel's and Martin's media. The Omeliansky's medium, being simpler in composition was selected for further investigations described herein.

Effect of indole-acetic acid on cellulolytic activity—The fungus was grown on Omeliansky's and Czapek-Dox's media (with cellulose as source of carbohydrate) supplemented by indole-acetic acid in various concentrations. The reducing sugars liberated by the fungus in the metabolic liquor at various periods of growth were determined. The cellulolytic activity of the metabolic liquor from Czapek-Dox's medium was also assessed after 17 days of growth. The results are given in Table III below:—

TABLE III

Effect of indole-3-acetic acid on production of reducing sugars and cellulolytic activity

Media	Concentration of indole-acetic acid*	Reducing sugars/4 ml of metabolic liquor expressed in mgm of glucose			Cellulolytic activity after 17 days growth (% hydrolysis of cellulose)
		Period of growth in days			
		7	17	21	
Czapek-Dox's medium	1 : 1000	..	0·12	..	11·0
	1 : 1000	..	0·09	..	17·5
	0	..	0·07	..	8·5
Omeliansky's medium	1 : 10,000	0·045	0·35	0·25	..
	1 : 50,000	0·015	0·18	0·10	..
	1 : 100,000	Nil	0·38	0·15	..
	0	Nil	0·14	0·08	..

* The ratio indicates parts of indole-acetic acid used in the medium.

The results in Table III show that the amount of reducing sugars produced increases with increase in the concentration of indole-acetic acid. The activity of the cellulolytic enzyme is maximum when the ratio of indole-acetic acid used in Czapek-Dox's medium is 1 : 1000.

Effect of different constituents of Omeliansky's medium on elaboration of enzyme—The effect of various constituents of Omeliansky's medium on elaboration of cellulolytic enzyme was studied. Seven different variations of constituents were selected. At different periods of growth, 10 ml. of the metabolic liquor were taken out and the same amount of sterilized medium was added

to keep the total volume of the medium constant. The enzyme activity of the metabolic liquor was assessed. The results are given in Table IV below:—

TABLE IV

Effect of different constituents of Omeliansky's medium on elaboration of the enzyme

Media	Cellulase activity (% hydrolysis of cellulose)						
	Set I Period of growth in weeks			Set II Period of growth in weeks			
	1	2	3	1	2	3	4
Omeliansky's medium 'O'	Nil	27·5	21·2	16·0	54·3	Nil	47·5
'O' with $(NH_4)_2HPO_4$ and without K_2HPO_4	Nil	28·8	28·8	6·0	38·5	10·5	5·0
'O' with KNO_3 and without K_2HPO_4	Nil	11·9	10·0	5·0	28·0	Nil	33·3
'O' with $NaNO_3$ and without K_2HPO_4	Nil	20·4	7·5	Nil	26·3	Nil	22·8
'O' with NH_4Cl and without $(NH_4)_2SO_4$	7·0	47·5	31·2	17·5	57·8	23·7	15·8
'O' with asparagine and without $(NH_4)_2SO_4$	Nil	17·0	Nil	16·0	43·8	7·0	13·8
'O' with $FeCl_3$ and without NaCl.	6·0	49·2	21·3	15·0	14·0	14·0	28·0
'O' without $MgSO_4$	7·0	13·6	5·0	15·0	33·3	Nil	Nil

On replacing $(NH_4)_2SO_4$ by NH_4Cl in the Omeliansky's medium, an improvement in the cellulolytic activity of the metabolic liquor was observed. In view of this improvement, most of the investigations reported in this paper have been carried out by this modified Omeliansky's medium.

Effect of different cellulosic substrates on the elaboration of the enzyme— The enzymatic activity of the metabolic liquor was assessed after 12—15 days of growth of the fungus on modified Omeliansky's medium, using the following cellulosic substrates:—

(*a*) Cellulose ethers (*i*) Cellofas A (methyl ethyl ether of cellulose, degree of substitution,—OCH_3=0·4 and—C_2H_5=0·9) and (*ii*) Cellofas B (Sodium salt of carboxymethyl cellulose, degree of substitution 0·45 to 0·55).

(*b*) Filter paper (Whatman No. 1).

The results are summarised in Table V below:—

TABLE V

Effect of different cellulosic substrates on the elaboration of the enzyme

No.	Substrate for growth	Period of growth (days)	Substrate for assay of cellulase activity	Cellulase activity (% hydrolysis of cellulose)	Cellobiase activity (% hydrolysis of cellobiose)
1	Filter paper	12	Cuprammonium cellulose	2·0	Not done
2	Cellofas 'A'	12	Cuprammonium cellulose	Nil	,,
3	Filter paper	12	Cellofas 'A'	Nil	,,
4	Cellofas 'A'	12	Cellofas 'A'	18·0	,,
5	Cellofas 'B'	12	Cuprammonium cellulose	11·2	,,
6	Cellofas 'B'	13	Cuprammonium cellulose	18·0	,,
7	Cellofas 'B'	15	Cuprammonium cellulose	3·6	,,
8	Cellofas 'A'	12	Cellofas 'B'	(*i*) 28·8 (*ii*) 16·1	(*i*) 36·6 (*ii*) 15·0
9	Cellofas 'B'	12	Cellofas 'B'	(*i*) 18·0 (*ii*) 11·3	(*i*) 33·0 (*ii*) 9·2

The data in Table V show that the fungus elaborates a highly active cellulolytic enzyme when Cellofas A and Cellofas B are used as cellulosic substrates.

Effect of subthreshold concentrations of sodium pentachlorophenate on elaboration of the enzyme—

(*a*) *Determination of subthreshold concentration*—Radial growth of the fungus on modified Omeliansky's medium containing different quantities of sodium pentachlorophenate was measured after different periods of growth. The results are given in Table VI below:—

TABLE VI

Effect of sodium pentachlorophenate on the growth of the fungus

Cellulosic substrate	Concentration of sodium pentachlorophenate (%)	Radial growth in cms — Period of growth in days								
		3	6	9	10	13	15	18	19	21
Cellofas 'A'	0·0001	Nil	3·22	4·04	..	5·12	5·12	..	5·88	5·88
	0·00025	Nil	2·65	2·95	..	2·95	2·95	..	3·7	3·7
	0·00062	Nil	2·4	2·62	..	2·63	2·63	..	2·63	2·63
	0·00156	Nil	1·43	1·78	..	1·82	1·83	..	1·93	1·93
	0·00391	Nil	Nil	Nil	..	Nil	Nil	..	Nil	Nil
	0·00977	Nil	Nil	Nil	..	Nil	Nil	..	Nil	Nil
	Control	Nil	3·36	5·42	..	7·5	8·1	..	8·98	8·98
Cellofas 'B'	0·001	1·16	1·95	..	2·13	3·31	4·4	6·38	..	..
	0·0015	1·0	1·58	..	1·75	1·85	1·95	1·95	..	..
	0·002	Nil	1·73	..	1·73	1·73	1·85	1·85	..	..
	Control	2·28	5·38	..	7·66	10·75	11·78	12·3	..	..

It is evident from the results given in Table VI that restricted growth takes place when 0·0015 and 0·002% of sodium pentachlorophenate were used.

The subthreshold concentration of the pentachlorophenate, therefore, lies round 0·0020%.

(*b*) *Effect of subthreshold concentrations of sodium pentachlorophenate in the growth medium on elaboration of the enzyme*—The fungus was grown for 12 days on modified Omeliansky's medium with Cellofas 'B' as source of carbohydrate and containing different concentrations of sodium pentachlorophenate. The enzyme activity was assessed using Cellofas 'B' and Cellobiose as substrates. The results are given in Table VII below:—

TABLE VII

Effect of subthreshold concentrations of sodium pentachlorophenate on elaboration of the enzyme

Concentration of sodium pentachlorophenate (%)	Cellulase activity (% hydrolysis of cellulose)		Cellobiase activity expressed in ml of N/200 sodium thiosulphate soln.
	Expt. I	Expt. II	
0·001 ..	11·50	7·4	9·52
0·0015 ..	0·92	..	..
0·002 ..	0·91	0·39	2·99
0·0025 ..	..	..	..
0·003 ..	..	..	0·17
0·004 ..	..	..	0·45
0·005 ..	..	..	0·45
Control (without fungicide) ..	7·84	4·8	6·10

It appears from the data in Table VII that just below the subthreshold concentration of pentachlorophenate i.e. at 0·001% the organism elaborates more active cellulolytic enzyme.

Discussion

Utilisation of cellulose as sole source of carbon—These investigations confirm that cellulase is not a constitutive enzyme but an adaptive one [10,11,12] since it is elaborated only in response to a specific substrate i.e. cellulose. It has been reported by Talboys [13] that substrates other than cellulose or cellobiose in the culture medium resist the elaboration of the cellulolytic enzyme by *Verticillium albo-atrum*. Youatt [14] working on the growth of *Stachybotrys atra* on Waksman —Carey medium reported the important role of cellulose used as the sole source of carbon. Glucose (and not fructose and xylose) was reported to inhibit the *in vivo* cellulolytic activity of *Sporocytophaga myxococcoides* by Sijpesteijn and Fåhreaus [15]. In case of *Myrothecium verrucaria* Sinden *et al* [2] also reported similar results by *in vivo* studies. During the present studies, it was found that the fungus does not elaborate the cellulolytic enzyme when sucrose is present in the medium either alone or with cellulose. The preferential utilisation of sucrose, a simpler carbon compound by the fungus may be inhibiting cellulolytic astivity. This has also been reported by Siu *et al* [16] during their work on the growth of *M. verrucaria* using a mixture of cellulose and sucrose.

Effect of different media—Both Omeliansky's and Fries' media gave an active cellulolytic enzyme. The two media were also quite suitable for growth requirements as assessed by mycelial weight, radial growth and cellulose-destroying index*.

* D.R.L.(S) Kanpur Bio Report No. Bio/60/91 (1960)

It is clear, however, that nutritional requirements for the growth of the fungus are not identical to those for elaboration of an active extra cellular cellulolytic enzyme. This can be explained as the former is resultant of a far more complex multi-enzyme system than the latter.

Effect of indole-acetic acid—This compound is known to be a plant hormone and an auxin [17, 18]. It is converted to a glutamine conjugate[19]. These investigations show that the enzyme elaborated in the presence of indole-acetic acid is more active; the amount of reducing sugars produced is also increased, indicating a higher cellulolytic activity *in vivo*. The cellulolytic activity *in vivo* has been found to be affected by a number of growth promoting substances [20]. The data, however, lack in the effect of these compounds on elaboration of extra-cellular enzymes.

Effect of different constituents of Omeliansky's medium—The replacement of ammonium sulphate by ammonium chloride in the culture medium increases the elaboration of the enzyme. This confirms the previous recommendation of Waksman and Bred [12]. It is further observed that (*a*) magnesium is an important constituent of the medium and its omission leads to elaboration of a weak cellulolytic enzyme (*b*) potassium proves better as phosphate than as nitrate and (*c*) asparagine is inferior to ammonium compounds as a source of nitrogen. Organisms differ in their requirements of sources of nitrogen. Literature survey shows that different species of the same genus react differently. Some organisms such as *Itersonia ferruginea* [22], *Sorangium compositum*[23], *S. nigrescens* [23] and *Aspergillus fumigatus* [24] grow better when nitrogen is supplied in the form of inorganic salts than in organic compounds such as peptone. Others such as *Fusarium* sp. [25] and *Humicola grisea*[24] prefer the reverse. The cellulolytic activity, however, in all these cases was determined *in vivo*.

Effect of cellulosic substrates—The results show that both Cellofas 'A' and Cellofas 'B' stimulate elaboration of cellulolytic enzyme as compared to other cellulosic substrates used. This may be due to the fact that soluble cellulose derivatives can be hydrolysed easily by intracellular enzyme which is active in initial stages of growth[1]. These results differ from those reported by Siu *et al*[26] on *in vivo* studies with *M. verrucaria* on different cellulosic substrates. It may be due to a different degree of substitution in cellulose derivatives used. It has been widely reported that degree of substitution of the cellulose derivatives plays an important role on the degradation of cellulose by micro-organism both *in vivo* [27] and *in vitro* [28].

Effect of subthreshold concentrations of sodium pentachlorophenate—0·001 to 0·003% of sodium pentachlorophenate inhibited the growth of the fungus. The fungus when grown in the presence of the inhibitor at subthreshold concentrations i.e. at 0·001% elaborated cellulolytic enzymes of enhanced activity. This may be due to vigorous activity of the living organism against adverse environments in their struggle for existence. No other instance of this nature is available in the literature.

Acknowledgement

The authors wish to thank Shri S. K. Ranganathan for helpful suggestions and interest in this investigation. Their thanks are also due to Dr. P. N. Agarwal, Assistant Director, Biology Division for valuable advice in preparing this paper for publication.

References

1. Verma, G. M., and Verma, R. K., *Def. Sci. J.* **12**, 285, 1962.
2. Sinden, J. W., Mix, A. J., and Siu, R. G. H., "Microbiological series Report No. 8", U.S. Army Biological Laboratories Philadelphia 1948.
3. Omeliansky W., *Centr. Bakt.*, II Abt, **8**, 226, 1902.
4. Greathouse, G.A., Klemme, D. E. and Barker, H. D., *Ind. and Engineering* Anal. Ed., **14**, 614, 1942.
5. Mandel's, G. R. and Norton, A.B., "Quartermaster General Laboratories Report No. 11", Philadelphia 1948.
6. Martin, G.W., "Report of Tropical Deterioration Administrative Committee" Washington 1945.
7. Ryan, F. J., Beadle, G. W. and Tatum, E. L., *Amer. J. of Botany*, **30**, 784, 1943.
8. Ainsworth, G. C. and Bisby, G. R., "A dictionary of the fungi" The Imperial Mycological Institute, p. 190, 1945.
9. Shaffer, P. A. and Somogyi, M., *J. Biol. Chem.*, **100**, 695, 1933.
10. Cowles, P.B. and Rettger, L. F., *J. Bact.*, **21**, 167, 1931.
11. Karstrom, H., *Ann. Acad. Sci. Fennicae.*, Sec. A, 33(2), 1930.
12. Karstrom, H., *Ergeb. Enzym forsch.*, **7**, 350, 1938.
13. Talboys, P. W., *Brit. Mycol. Soc. Trans.*, **41**, 242, 1958.
14. Youatt, G., *Australian J. Biol. Sci.*, **11**, 209, 1958.
15. Sijpesteijn, A. K. and Fåhraeus, G., *J. Gen. Microbiol.*, **3**, 224, 1949.
16. Siu, R. G. H., Saunders, P. R., Genest, R., and Dangey, J., "Tropical Report No. 1" U.S. Army Biological Laboratories, Philadelphia.
17. Porter, J. R., "Bacterial Chemistry and Physiology" John Wiley & Sons Inc. New York, p. 759, 1946.
18. Jensen, P. B., "Growth hormones in Plants" McGrawhill Book Company Inc. New York & London, p. 41, 1936.
19. Williams, R. T., "Deterioration Mechanisms", Chapman & Hall Ltd., p. 671, 1959.
20. Reese, E. T., Thesis, State College Pennysylvania, 1946.
21. Fred, E. B. and Waksman, S. A., "Laboratory Manual of Microbiology" Mc-Graw Hill Book Company Inc. New York & London, p. 35, 1928.
22. Rippel, A. and Flehming, T., *Arch. Microbiol.*, **4**, 229, 1933.
23. Krzemieniowskii, H. and S., *Bull. Intern. Acad. Polon. Sci. Classe. Sci. Math. nat.*, B. **33**, 1937.
24. Dawson, A. I., *J. Bact.*, **4**, 133, 1919.
25. Heller, F., *Zentr. Biochem. Biophys.*, **21**, 404, 1919.
26. Siu, R. G. H., Darby, R. T., Burkholder, P. R. and Barghoorn, E. S., *Textile Research, J.*, **19**, 484, 1949.
27. Burkholder, P. R., and Siu, R. G. H., "Tropical Report No. 23", U.S. Army Biological Laboratories, Philadelphia 1947.
28. Reese, E. T., Siu, R. G. H. and Levinson, H. L., *J. Bact.*, **59**, 485, 1950.