Biochemical Pharmacology, Vol. 25, pp. 2091–2095, Pergamon Press, 1976. Printed in Great Britain.

INHIBITORY EFFECTS OF MYCOTOXINS ON Na^+-DEPENDENT TRANSPORT OF GLYCINE IN RABBIT RETICULOCYTES

YOSHIO UENO, HIROKO MATSUMOTO, KAZUKO ISHII and KEN-ICHI KUKITA

Laboratory of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Ichigaya, Tokyo 162, Japan

(*Received* 12 *September* 1974; *accepted* 11 *February* 1976)

Abstract—An inhibitory effect of toxic fungal metabolites on Na^+-dependent transport of [^{14}C]glycine by rabbit reticulocytes was examined. Among twenty-three mycotoxins tested, patulin, a carcinogenic lactone of *Penicillium* and *Aspergillus*, caused the most marked inhibition. From comparative experiments with several metabolic inhibitors and SH-blockaders, patulin was presumed to inactivate SH groups of the receptor site for the glycine transport system in reticulocyte membranes.

Toxic fungal metabolites (so-called mycotoxins), which exhibit carcinogenicity and cytotoxicity, are recognized as naturally occurring pollutants in foods and feeds; it is desirable, therefore, to develop sensitive and specific methods for biological detection of mycotoxins. In preceding papers, the authors reported that, among mycotoxins tested, carcinogenic anthraquinones such as (−)luteoskyrin and (+)rugulosin from *Penicillium* spp. inhibited selectively cellular multiplication of *Tetrahymena pyriformis* GL [1], and *Escherichia coli* F-11 which have defective cell-walls [2], that carcinogenic mycotoxins such as aflatoxins and sterigmatocystins inhibited the cellular growth of a recombination-deficient mutant of *Bacillus subtilis* [3], and furthermore, that cytotoxic trichothecenes such as fusarenon-X and T-2 toxin from *Fusarium* spp. were found to inhibit the synthesis of protein in rabbit reticulocytes [4–7]. These biological assay methods were employed for screening toxic metabolites and toxin-producing fungi.

In the present paper, employing rabbit reticulocytes as an assay tool, the authors attempt to select and identify the mycotoxins which interfere with transport systems of amino acids. The reticulocytes have been shown to be capable of concentrating glycine and alanine in the presence of extracellular [Na^+] [8]. The results indicate that patulin, a carcinogenic lactone produced by *Penicillium* and *Aspergillus* spp., impairs the Na^+-dependent glycine transport system of reticulocytes.

MATERIALS AND METHODS

Reticulocytes were prepared from rabbits as reported elsewhere [4]; for experiments involving Na^+-free media, the cells were washed with ice-cold choline chloride (154 mM) and Tris–HCl buffer (170 mM, pH 7.6), and the packed cells were then suspended in 2 vol. of the buffer. The rate of uptake of amino acids was measured by the use of ^{14}C-labeled amino acids, essentially as described by Wheeler and Christensen [8], as follows. The rabbit reticulocytes were incubated for 45 min at 37° in media containing Tris–HCl (17 mM, pH 7.6), $MgSO_4$ (1 mM), KCl (5 mM), [^{14}C]glycine (0.2 mM) and NaCl (135 mM). After the incubation, 1 vol. of ice-cold Tris–HCl buffer was added, the mixture was centrifuged at 2000 rev/min for 5 min, and the packed cells were extracted with 1 ml of 20% trichloroacetic acid. To vials containing 10 ml of a standard dioxane–naphthalene scintillating solution, 0.2 ml of the supernatant of the acid-soluble fraction was added.

Chromatographic analysis of acid extracts from incubated cells was carried out to determine whether metabolic changes of labeled amino acid occurred during the incubation. Ascending chromatography on Toyo-roshi No. 51A paper (2 × 40 cm) was used with *n*-butanol–acetic acid–water (4:1:1, by vol.). The dried chromatograms were cut into strips (2 × 1 cm) and placed in vials containing 10 ml of a standard toluene scintillating solution.

The uptake was expressed as the ratio of [^{14}C] in cell water divided by that in the incubation medium ($[^{14}C]_{cell\ water}/[^{14}C]_{medium}$). Cell water content was determined by drying packed cells to constant weight at 110° under reduced pressure. The water content of rabbit cells containing 70–90 per cent reticulocytes was estimated to be 73 per cent.

Patulin added to the reaction solution was photometrically determined as follows. The reaction mixture was centrifuged at 3000 rev/min for 3 min, the supernatant was extracted twice with 1 ml ethylacetate, and the combined solvent was evaporated to dryness. The residue was dissolved into 5 ml water followed by the addition of 1 ml of 4% phenylhydrazine·HCl. After heating the mixture at 100° for 10 min, the optical density was measured at 470 nm.

Mycotoxins were isolated from culture filtrates or fungal mats of the following fungi according to the methods already reported: (−)luteoskyrin and chlorine-containing peptide (Cl-peptide) from *Penicillium islandicum* [9, 10]; (+)rugulosin from *P. rugulosum* [11]; citreoviridin from *P. citreo-viride* [12]; patulin from *P. patulum*; penicillic acid from *P. olivino-viride*; citrinin from *P. citrinum*; fusarenon-X from *Fasarium nivale* [13, 14]; T-2 toxin, neosolaniol and butenolide from *F. solani* and *F. sporotrichioides* [15]; and zearalenone from *F. roseum* [16]. Sterigmatocystin and

ochratoxin A, chaetoglobosin A, and ascradiol and fusaric acid were generously supplied by Drs. Yamazaki (Chiba University), Natori and Tanabe (National Institute of Hygienic Sciences) and Nishimura (Tottori University) respectively. Moniliformin and desoxypatulinic acid were obtained from R. J. Cole (U.S.A.) and P. M. Scott (Canada). Aflatoxin B_1, rubratoxin B, and cytochalasin A and B were the products of Makor Chemical Ltd. and Aldrich Chemical Co. Inc.

For experiments, water-soluble mycotoxins were dissolved in distilled water, and water-insoluble agents, such as aflatoxin B_1, sterigmatocystin and zearalenone, were dissolved in ethanol or dimethylsulfoxide. (−)Luteoskyrin, (+)rugulosin and ochratoxin A were dissolved in water and neutralized with Tris (hydroxymethyl)-aminomethane to pH 7.

[1-^{14}C]glycine (20 mCi/m-mole) and [1-^{14}C]alanine (10 mCi/m-mole) were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo) and the Radiochemical Centre Ltd. (Amersham), respectively, and the radioactivity was determined in a liquid scintillation spectrometer (Aloka LSC-502).

Details for individual experiments are recorded in the legends of the tables and figures.

RESULTS

Inhibitory effects of mycotoxins on uptake of [^{14}C]glycine by reticulocytes. As reported by Wheeler and Christensen [8], uptake of glycine and alanine by reticulocytes depends on extracellular ions, and the rate of uptake is stimulated by Na^+. This finding was confirmed as follows. In the absence of extracellular Na^+, the intracellular glycine increased to a saturation concentration which corresponded to a ratio ($[^{14}C]_{cell\ water}/[^{14}C]_{medium}$) of 1. The rate of uptake was stimulated by replacing extracellular choline chloride with NaCl, and the maximum ratio reached 4–5 after incubation for 45 min. Chromatographic analysis of the acid-soluble fraction of the cells revealed that over 95 per cent of the total radioactivity transported into the cells was derived from [^{14}C]glycine itself, and no detectable amount of the radioactivity was found in the acid-insoluble fraction of the cells, either in the absence or presence of external Na^+.

These results indicate that, in the absence of external Na^+, the uptake of glycine by reticulocytes proceeds to a concentration equilibrated between intra- and extracellular fluids, and in the presence of Na^+, glycine in the medium is actively transported into the cells. Similar results were obtained with [^{14}C]alanine.

Effects of mycotoxins on the transport of glycine. Twenty-three toxic fungal metabolites were tested for their inhibitory effects on the transport of [^{14}C]glycine in the presence and absence of external Na^+. No significant effect of the solvents of mycotoxins on the uptake was observed up to a final concentration of 0.5% ethanol and 0.1% dimethylsulfoxide; the transport was slightly stimulated in the presence of 5% ethanol and 1% dimethylsulfoxide.

The inhibitory effects of seven *Fusarium* toxins (fusarenon-X, T-2 toxin, neosolaniol, butenolide, zearalenone, fusaric acid and moniliformin), ten *Penicillium* toxins [(−)luteoskyrin, (+)rugulosin, chlorine-containing peptide, citrinin, citreoviridin, penicillic acid, rubratoxin B, patulin, deoxypatulinic acid and ascradiol], three *Aspergillus* toxins (ochratoxin A, aflatoxin B_1 and sterigmatocystin), and three others (chaetoglobosin A, and cytochalasin A and B) were examined.

In the absence of external Na^+, the mycotoxins caused no significant effects on the transport of [^{14}C]glycine up to 100 μg/ml of mycotoxins. In the presence of Na^+, patulin and Cl-peptide inhibited the transport of glycine. The dose response curve of patulin, as shown in Fig. 1, revealed that 5, 10 and 100 μg/ml of patulin produced 10, 25 and 100 per cent inhibition, respectively, and the concentration of patulin causing 50 per cent inhibition was estimated to be 30 μg/ml (2×10^{-4} M). The Cl-peptide caused an inhibition of 30 per cent at a concentration of 100 μg/ml.

No significant inhibition occurs with 100 μg ml of the following mycotoxins: fusarenon-X, T-2 toxin, neosolaniol, butenolide, zearalenone, fusaric acid, moniliformin, luteoskyrin, rugulosin, citrinin, citreoviridin, penicillic acid, rubratoxin B, ochratoxin A, aflatoxin B_1, sterigmatocystin, chaetoglobosin A, and cytochalasin A and B.

Inhibitory effects of metabolic inhibitors. The transport of amino acids by biomembranes is controlled by several metabolic processes of cells such as energy production, cation transport and others. To clarify the mechanism of active transport of glycine by reticulocytes and the inhibitory mode of action of patulin observed above, the authors examined the effects of several metabolic inhibitors, antibiotics, and SH-blocking agents on the passive and active transport of glycine by the reticulocytes.

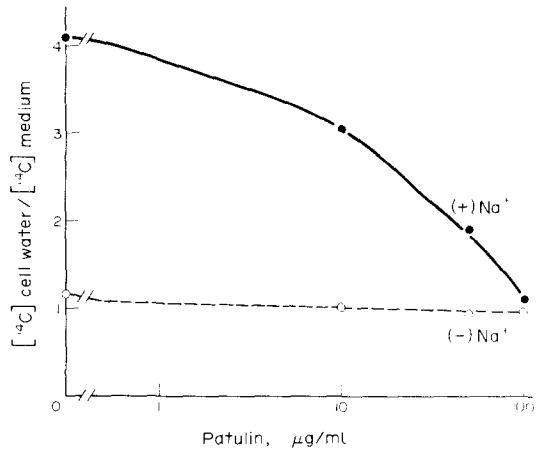

Fig. 1. Dose response curve of patulin. A solution containing 0.1 ml of a 30% reticulocyte suspension, 17 μmoles Tris-HCl buffer (pH 7.6), 5 μmoles KCl, 1 μmole $MgSO_4$, 0.2 μmole [^{14}C]glycine (0.05 μCi) and the desired amount of patulin, in a total volume of 1.0 ml, was incubated at 37° in the presence of 135 μmoles NaCl (● ● ●), or 135 μmoles choline choride (○---○). After incubation for 45 min, 1 vol. of ice-cold Tris-HCl buffer (170 mM, pH 7.6) was added and the mixture centrifuged for 5 min at 2000 rev/min. The supernatant solution was drawn off immediately with a pipette, and 1 ml of ice-cold 20% trichloroacetic acid solution was added to the packed cells. After centrifugation for 5 min at 2000 rev/min, 0.2-ml portions of the clear supernatant solutions were analyzed for radioactivity.

Table 1. Inhibition of glycine transport by SH-blockaders

SH-blockaders	Concn (10^{-5} M)	$[^{14}C]_{cell\ water}/[^{14}C]_{medium}$	Inhibition (%)
		4.7	0
Phenylmercury acetate	3	2.5	60
p-Chloromercury benzoate	2	2.7	65
3,6-(2,5)-bis(Acetate-mercurimethyl)-*p*-dioxane	8	3.2	40
n-Ethylmaleimide	80	1.0	100

The three potent inhibitors of respiration and its coupled phosphorylation were: antimycin, oligomycin and NaN_3. No effect of the inhibitors was demonstrated at concentrations up to 100 μg/ml. g-Strophanthin and phlorizin, potent inhibitors of active cation and sugar transport, also failed to inhibit the Na^+-dependent transport of glycine. Of four inhibitors of protein synthesis, puromycin, cycloheximide, streptomycin and chloramphenicol, the last compound produced an inhibition of uptake of 45 per cent at a concentration of 160 μg/ml (0.1 mM).

The inhibitory effects of organic mercury compounds and *N*-ethylmaleimide are summarized in Table 1. Inhibitory effects of organic mercury compounds were examined below 10^{-4} M because of their hemolytic property. The results reveal that all of the mercury compounds and *N*-ethylmaleimide produce a significant inhibition of the transport only in the presence of external Na^+.

Inhibitory mechanism of patulin. The kinetics of the inhibitory effect of patulin on the glycine transport were analyzed. The time course of the transport revealed, as shown in Fig. 2, that Na^+-dependent transport of glycine into the cells was inhibited by patulin in parallel to the incubation time up to 40 min, and an additional incubation for 60 min resulted in the decrease of the ratio ($[^{14}C]_{cell\ water}/[^{14}C]_{medium}$). This finding indicates that patulin inhibits the influx of amino acid in the early phase of incubation and stimulates the outflow in the latter.

As for the influx of glycine, the inhibitory effect of patulin was not reversed by increasing the external concentration of Na^+ (Fig. 3).

A Lineweaver–Burk plot showed that the K_m value for glycine was 1.33 mM at 33 mM Na^+ and 0.69 mM at 135 mM Na^+, and the V_{max} was estimated to be 0.19 μmoles/15 min. In the presence of 100 μg/ml (6.5×10^{-4} M) of patulin, the K_m values for glycine were the same as above at the two concentrations of external Na^+; the V_{max} was changed to 0.16 μmoles/15 min. This result indicates that the mycotoxin inhibits non-competitively the influx of glycine (Fig. 4).

Further experiments with SH-compounds revealed that the inhibitory effect of patulin was prevented by a prior addition of dithiothreitol or glutathione to the incubation system; complete recovery was obtained by equimolar amounts of these SH-compounds (Fig. 5). In contrast, when the cells preincubated with 3.2×10^{-4} M of patulin for 25 min were subjected to incubation in the complete system, the reduced transport of glycine was not reversed by

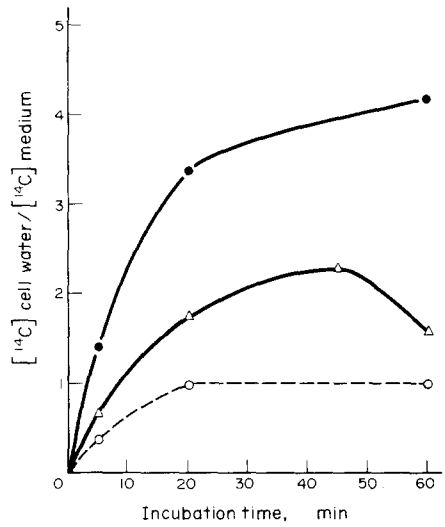

Fig. 2. Time course of the inhibitory effect of patulin. The reticulocytes were incubated in the presence (●——●) or absence (○---○) of NaCl as described in Fig. 1, and 50 μg/ml of patulin was added to the reaction mixture containing NaCl (△——△).

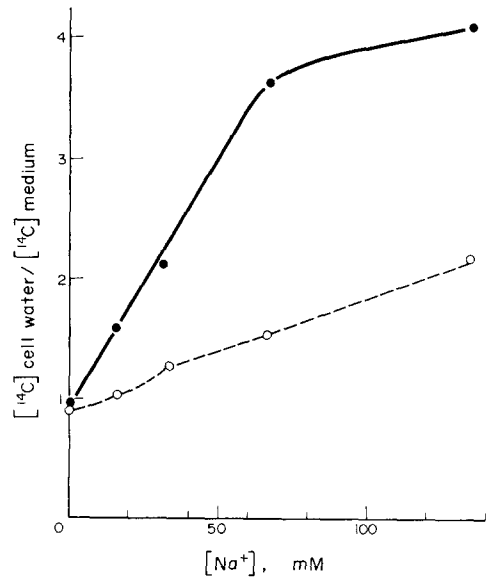

Fig. 3. Effect of Na^+ concentration on the inhibition of glycine transport by patulin. Key: (●——●) control; and (○---○) 50 μg/ml of patulin.

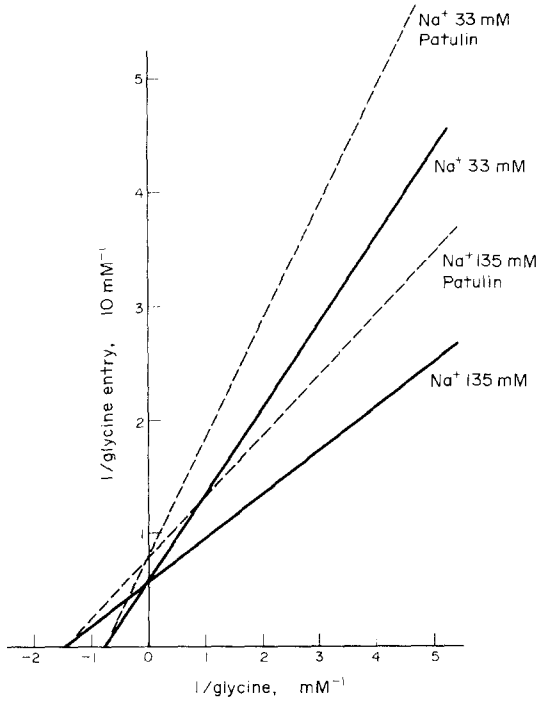

Fig. 4. Lineweaver–Burk plot of glycine transport. The reticulocytes were incubated for 15 min in the presence or absence of 100 μg/ml of patulin.

addition of an excess amount of glutathione (Table 2).

Photometric determination of patulin revealed that, as shown in Table 3, the recovery of the mycotoxin from the medium was only 60 per cent, irrespective of the presence or absence of external Na^+. This indicates that around 40 per cent of patulin added to the incubation medium binds with cellular components.

DISCUSSION

The results described above show that, among 23 mycotoxins tested, patulin (Fig. 6) was found to be a potent inhibitor of Na^+-dependent transport of glycine by rabbit reticulocytes. Patulin is reported to be antibacterial, mutagenic to yeast and phytotoxic [17]. Repeated subcutaneous injections induced sarcoma in rats [18].

Chemically speaking, patulin is one of the carcinogenic lactone compounds, and the lactone ring is assumed to be responsible for their toxicity as well as their carcinogenicity [19]. In the present results, however, other carcinogenic lactones, such as penicillic acid, aflatoxin B_1 and ochratoxin A, did not inhibit the transport of glycine. Furthermore, the lack of inhibitory effects of ascradiol and desoxypatulinic acid suggests that the hydroxyl group at C-4 of patulin plays an important role in its inhibitory activity. As for Cl-peptide, this mycotoxin is highly toxic to animals, causing vacuolation, edema and swelling of the liver tissue [20] and the author found impairment of glycogen metabolism in the liver [21]. Itoh *et al.* [22] reported that disturbance of the circulation of capillaries in the liver may be responsible for the damage to the liver by the peptide. The relation of these results to those biological features of the peptide remains to be clarified. It should be noticed that the peptide and chloramphenicol, both having two chlorine atoms, are inhibitory to the transport system.

As a step in disclosing the mode of action of patulin, the authors attempted to clarify the mechanism of Na^+-dependent active accumulation of glycine by the reticulocytes. As far as the action of Na^+ is concerned, we have considered the possibility of the Na^+-

Table 2. Protective effect of glutathione on the inhibition of glycine transport by patulin

Conditions	$[^{14}C]_{cell\ water}/[^{14}C]_{medium}$
Control	5.4
Plus patulin*	2.5
Plus glutathione†	4.9
Cells preincubated with patulin were washed and incubated in the absence of glutathione	1.7
Cells preincubated with patulin were washed and incubated in the presence of glutathione	2.9

* Patulin: 3.2×10^{-4} M.
† Glutathione: 1.6×10^{-3} M.

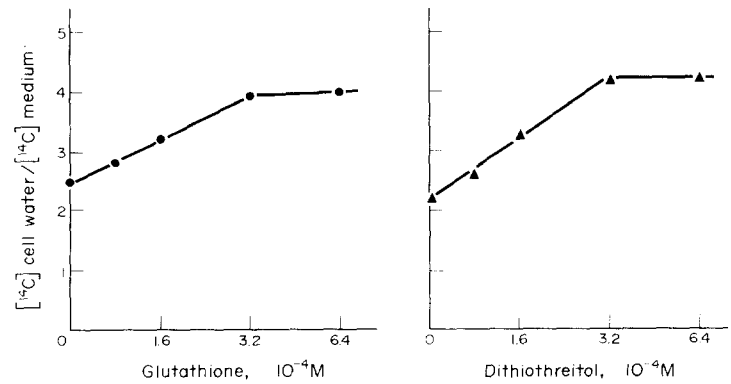

Fig. 5. Protection of the inhibitory effect of patulin by SH-compounds. The reticulocytes were incubated for 45 min in the presence of 50 μg/ml (3.2×10^{-4} M) of patulin; glutathione or dithiothreitol was added to the reaction mixture prior to patulin.

Table 3. Photometric determination of patulin*

Incubation time (min)	Na^+	Patulin in the medium (μg/ml)
0	−	88
0	+	90
45	−	57
45	+	60

* The reaction mixture (1 ml) containing 100 μg patulin was incubated at 37° in the presence or absence of Na^+.

dependent transport of amino acid (a) being directly linked to active cation transport in the cells or (b) being mediated by a carrier system which is active in the presence of a favorable [Na^+] gradient across the cell membrane. Lack of inhibition of the uptake by cation-transport inhibitors such as g-strophanthin and oligomycin suggests that no direct correlation with active cation transport exists.

The present results obtained with SH-blocking agents (Table 1) suggest that SH-groups are part of the active center of the carrier system of the cell membrane. From the data on the kinetic analysis (Figs. 3 and 4), the irreversible inactivation (Table 2), and the chemical analysis of patulin (Table 3), we are convinced that patulin binds irreversibly to the SH-groups of the cell membrane. Therefore, it is highly possible that the masking of SH-groups on the center fails to keep a favorable [Na^+] gradient across the cell membrane.

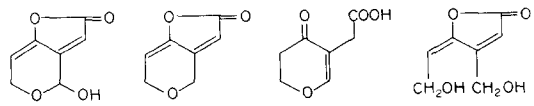

Fig. 6. Chemical structures of patulin and its derivatives.

Acknowledgements—The present investigation has been carried out, in part, with financial aid from the Ministry of Education for the advancement of cancer research (1973).

REFERENCES

1. Y. Ueno and M. Saheki, *Jap. J. exp. Med.* **38**, 157 (1968).
2. H. Yamakawa and Y. Ueno, *Chem. pharm. Bull., Tokyo* **18**, 177 (1970).
3. Y. Ueno and K. Kubota *Cancer Res.* **36**, 445 (1976).
4. Y. Ueno, M. Hosoya and Y. Ishikawa, *J. Biochem., Tokyo* **66**, 419 (1969).
5. Y. Ueno, M. Nakajima, K. Sakai, K. Ishii, N. Sato and N. Shimada, *J. Biochem., Tokyo* **74**, 285 (1973).
6. Y. Ueno, N. Sato, K. Ishii, H. Tsunoda and M. Enomoto, *Appl. Microbiol.* **25**, 699 (1973).
7. Y. Ueno and N. Shimada, *Chem. pharm. Bull., Tokyo* **22**, 2744 (1974).
8. K. P. Wheeler and H. N. Christensen, *J. biol. Chem.* **242**, 1450 (1967).
9. Y. Ueno and I. Ishikawa, *Appl. Microbiol.* **18**, 406 (1969).
10. K. Ishii and Y. Ueno, *Appl. Microbiol.* **26**, 359 (1973).
11. Y. Ueno, I. Ueno, N. Sato, Y. Iitoi, M. Saito, M. Enomoto and H. Tsunoda, *Jap. J. exp. Med.* **41**, 177 (1971).
12. Y. Ueno and I. Ueno, *Jap. J. exp. Med.* **42**, 91 (1972).
13. Y. Ueno, I. Ueno, T. Tatsuno, K. Ohokubo and H. Tsunoda, *Experientia* **25**, 1062 (1969).
14. Y. Ueno, I. Ueno, K. Amakai, Y. Ishikawa, H. Tsunoda, K. Okubo, M. Saito and M. Enomoto, *Jap. J. exp. Med.* **41**, 507 (1971).
15. Y. Ueno, N. Sato, K. Ishii, K. Sakai and M. Enomoto, *Jap. J. exp. Med.* **42**, 461 (1972).
16. K. Ishii, M. Sawano, Y. Ueno and H. Tsunoda, *Appl. Microbiol.* **27**, 625 (1974).
17. A. Ciegler, R. W. Detroy and E. B. Lillehoj, in *Microbial Toxins* (Eds. A. Ciegler, S. Kadis and S. J. Ajl.). Vol. VI, p. 409. Academic Press, New York (1971).
18. C. Dickens and H. E. H. Jones, *Br. J. Cancer* **15**, 85 (1961).
19. C. Dickens, in *Potential Carcinogenic Hazard from Drugs* (Ed. R. Truhaut), p. 144. Springer, Berlin (1967).
20. K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, O. Yonemitsu, H. Itoh, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, *Jap. J. exp. Med.* **31**, 19 (1961).
21. Y. Ueno, M. Kaneko, T. Tatsuno, I. Ueno and K. Uraguchi, *Jap. J. biochem. Soc., Tokyo* **38**, 687 (1966).
22. H. Itoh, S. Yamasawa, T. Yamaguchi and Y. Horiguchi, *Yokohama med. Bull.* **14**, 1 (1963).