sence of exogenous substrate protein (such as histone) was noted. In contrast, about 36% inhibition in the absence of Mg^{2+} and 47% inhibition in the presence of Mg^{2+} on cyclic AMP-dependent protein kinases by this acidic protein was observed when arginine-rich histone was added to reaction mixture as a substrate. Such inhibition may be partially due to the strong interaction between histone and this acidic protein which may deprive a part of histone from phosphorylation by these kinases. Cyclic AMP-dependent protein kinases have been found in many mammalian tissues[15], including brain. Therefore, the results of this study suggest that this acidic protein may also play an important regulatory role on this type of kinases in brains. It is of great interest that the small acid protein stimulates both enzymes, phosphoprotein phosphatases and megamodulin-dependent protein kinase I, which in general carry out opposite functions. Therefore, the time sequence for the activation of both enzyme in vivo may be different to avoid antagonism.

This acidic protein is a heat-stable factor since it retained original stimulatory effect on the same enzymes after being boiled at 100 °C for 30 min. However, its stimulatory activity was destroyed after being treated with trypsin at 37 °C for 2 h, indicating that it indeed is a protein. Moreover, altered UV spectra of this acidic protein in the presence of Mg^{2+} or Mn^{2+} were noted, suggesting that the concomitant conformational transition[16] of this protein may occur through its binding with the cation to convert into its active forms, acidic protein-cation complexes, which may thereby modulate enzymes.

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2 Lin, Y.M., Molec. cell. Biochem. *45* (1982) 101.
3 Lin, Y.M., Liu, Y.P., and Cheung, W.Y., J. biol. Chem. *249* (1974) 4943.
4 Kuo, W.N., Cytobiose *36* (1983) 175.
5 Shoji, M., Brackett, N.L., Tse, J., Shapira, M., and Kuo, J.F., J. biol. Chem. *253* (1978) 3427.
6 Kuo, W.N., Biochem. biophys. Res. Commun. *114* (1983) 403.
7 Itano, T., Itano, R., and Penniston, J.T., J. Biochem. *189* (1980) 455.
8 Kuo, W.N., Experientia *39* (1983) 60.
9 Jamieson, G.A., Jr, and Vanaman, T.C., Biochem. biophys. Res. Commun. *90* (1979) 1048.
10 Kuo, W.N., unpublished observation.
11 Maeno, H., and Greengard, P., J. biol. Chem. *247* (1972) 3269.
12 Appleman, M.M., Birnbaumer, L., and Terres, H.N., J. biol. Chem. *116* (1966) 39.
13 Weber, K., and Osborn, M., J. biol. Chem. *244* (1969) 4406.
14 Winter, A., Ed, K., and Anderson, U.B., LKB Application Note 250 (1977).
15 Pasternak, Th., Cyclic AMP. Eds Robinson, Butcher, and Sutherland (1971) 127.

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Effects of diflubenzuron and tunicamycin on N-acetylglucosaminyl transferases in prepupae of the stable fly *(Stomoxys calcitrans)*

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Summary. Tunicamycin, an antibiotic, and diflubenzuron, an insect growth regulator, were tested to determine their effects on N-acetylglucosaminyl transferase from *S. calcitrans* prepupae. Diflubenzuron had no effect, but tunicamycin inhibited the transfer of GlcNAc-1-P from UDP-GlcNAc to dolicholmonophosphate with an I_{50} of 1.5–4 ng/ml.
Key words. Stable fly; *Stomoxys calcitrans;* diflubenzuron; tunicamycin; N-acetylglucosaminyl transferase; prepupae.

Chitin synthesis inhibitors (CSI) such as diflubenzuron have been under investigation for a number of years now. Reports concerning the molecular mode of action of CSI are often conflicting and contradictory[1]. Initially, CSI were thought to inhibit chitin synthesis in insects by acting directly on chitin synthase[2–5]. There are now several reports that show that CSI do not operate on chitin synthase when studied in cell-free enzyme preparations[1,6,7].

However, chitin synthesis is inhibited by diflubenzuron (DFB) and other CSI when in vitro organ culture or in vivo assays are used[2–5,8,9], and a buildup of UDP-N-acetylglucosamine (UDP-GlcNAc) is observed in tissues or insects that have been treated with CSI. In addition, Meola and Mayer[9] and DeLoach et al.[10] showed that DFB acted as a cytostatic agent when topically applied to pupae of the stable fly. Consequently, it was suggested that the inhibition of chitin synthesis and buildup of UDP-GlcNAc in DFB-treated tissues and the cytostatic action of DFB might be explained by an effect on cell membrane permeability[1,10]. Indeed, recent studies with Harding-Passey melanoma cells showed that DFB significantly inhibited the uptake of certain nucleosides into those cells, which indicated that a membrane effect was involved[11]. It has been suggested that DFB might inhibit the N-acetylglucosamine-1-P (GlcNAc-1-P) transferases which transfer GlcNAc-1-P from UDP-GlcNAc to form GlcNAc-pyrophosphoryl-dolichol (Dol·PP-GlcNAc), and which are involved in membrane synthesis[1]. Inhibition of this enzyme system could alter membrane permeability and cause a buildup of UDP-GlcNAc.

We have investigated the GlcNAc-1-P transferases in prepupae of stable fly, and report here the effects of DFB on these enzymes. The present studies were done in conjunction with studies on the antibiotic tunicamycin, which has been reported to inhibit chitin[12] and glycoprotein[13,14] synthesis in insects. Our results are the first demonstration that tunicamycin inhibits insect GlcNAc-1-P transferases to prevent the formation of Dol·PP-GlcNAc, as it does in other organisms.

Materials and methods. Chemicals. UDP-[glucosamine-6-^{3}H]GlcNAc (24 Ci/mmole) and UDP-[glucosamine-1-^{14}C]GlcNAc (35 mCi/mmole) were purchased, respectively, from New England Nuclear, Boston, MA and ICN Pharmaceuticals, Inc., Irvine, CA. Betafluor scintillation cocktail was purchased from National Diagnostics, Somerville, N.J. Dolichol monophosphate (Dol·P), 80–90% pure based on phosphorus content, was purchased from Sigma Chemical Co., St. Louis, MO. A purified sample of tunicamycin was obtained from Dr J.D. Douros, Developmental Therapeutics Program, Chemotherapy, NCI, Bethesda, MD. Diflubenzuron (Dimi-

lin®, TH-6040; N-[[(4-chlorophenyl)-amino]-carbonyl]-2,6-difluorobenzamide, 99% pure, was a gift of DuPhar, B.V., Holland.

Enzyme preparation. The enzyme was obtained from prepupae of stable flies reared according to the procedures of Mayer et al.[15]. Prepupae were homogenized in 4 vol. of 50 mM MOPS buffer (pH 7.5) containing 2.5 mM dithiothreitol (DTT), 0.25 M sucrose and 1 mM EDTA. Homogenates were strained through four layers of cheesecloth, then subjected to a differential centrifugation scheme to obtain the 10,000 × g pellet that was the enzyme source[7]. Final resuspension of the pellet was in 50 mM MOPS (pH 7.5) containing 2.5 mM DTT and 2 mM $MgCl_2$. The concentration of suspended protein was assayed by the method of Bradford using a gamma globulin standard, and then adjusted to 15 mg protein/ml.

Enzyme assay. The transfer of GlcNAc and GlcNAc-1-P from UDP-GlcNAc to Dol·P was measured using either ^{14}C or ^{3}H labeled UDP-GlcNAc.

Generally, the reaction mixtures contained 40 µg Dol·P, 20 µl 2% Triton X-100 in buffer, 265 µl 50 mM MOPS (pH 7.5), 0.8 µmole $MgCl_2$, 1 µmole DTT and 100 µl of enzyme (1.5 mg protein). Dol·P was suspended by sonicating the reaction mixture for 5 sec at 100 w, using a Braun-sonic sonicator equipped with a micro-probe, prior to the addition of enzyme. DFB (10^{-3}M) in dimethylsulfoxide (DMSO) and tunicamycin (2 mg/ml) in 0.001 N NaOH were serially diluted to the desired concentration in DMSO (DFB) or 50 mM MOPS (pH 7.5; tunicamycin). DFB was added to the reaction mixture in 1 µl amounts of DMSO and tunicamycin was added in 1–5 µl amounts of buffer 10 min prior to initiating the reaction. The reactions were initiated by adding substrate, either 70,000 cpm ^{3}H-UDP-GlcNAc or 150,000 cpm ^{14}C-UDP- GlcNAc. The radiolabeled substrate was first dried to remove ethanol and then dissolved in buffer before adding it to the reaction mixture. Reactions were conducted at 30 °C for 15 min.

Product analysis. The reactions were terminated by adding 4 ml $CHCl_3$:CH_3OH (1/1) and 1.4 ml water. These mixtures were shaken, then centrifuged for 1 min at 1000 × g to break the emulsion. The lower phases were removed to other test tubes and the upper phase extracted with 2 ml $CHCl_3$ as described above. The lower phase was removed and combined with the first extraction. The combined organic phases were then washed with 2.5 ml $CHCl_3$:CH_3OH:H_2O (3/48/47), vortexed, then centrifuged. To remove interfering neutral lipid products, the lower phases were then added to test tubes containing 200 mg (dry weight) DEAE cellulose that previously had been washed once with 6 ml $CHCl_3$:CH_3OH (2/1), twice with 4 ml 0.1 M ammonium acetate in CH_3OH, three times with 4 ml CH_3OH, and twice with 4 ml $CHCl_3$/CH_3OH (2/1). The organic phases were mixed with the DEAE cellulose, then centrifuged at 2000 × g for 2 min. Afterwards the supernatant was removed by aspiration. The DEAE cellulose was then washed sequentially four times with 4 ml $CHCl_3$:CH_3OH (2/1) and four times with 4 ml CH_3OH. The DEAE cellulose was vortexed after each solvent addition and centrifuged as before. The supernatant was removed by aspiration. The mono- and disaccharide substituted Dol·PP (carbohydrate analysis and other product characterization will be reported elsewhere) was then removed from the DEAE cellulose by washing three times with 4 ml 0.1 M ammonium acetate dissolved in CH_3OH. After vortexing and centrifugation, the supernatants were either added directly to scintillation vials or pooled in 300 ml round bottom flasks to be processed for TLC analysis. Samples collected in scintillation vials were placed in a fume hood overnight, where the solvent was evaporated by air drying. 10 ml of Betafluor scintillation cocktail was added to each vial and the radioactivity measured in a scintillation spectrometer.

Solvent was removed from samples to be used for TLC analysis by evaporation in vacuo on a rotary evaporator. To remove ammonium acetate, the residue was transferred to 125-ml separatory funnels by washing the flasks with 40 ml $CHCl_3$/CH_3OH (2/1), then 16 ml H_2O. The $CHCl_3$ layer was removed and dried. The residue was redissolved in 40 ml $CHCl_3$/CH_3OH (2/1) and washed a second time with 16 ml H_2O. The $CHCl_3$ layer was removed and dried under vacuum on a rotary evaporator. Residues were dissolved in $CHCl_3$:CH_3OH (2/1) and spotted on TLC plates (20 × 20 cm) coated with 0.25 mm silica gel G (Analtech, Newark, DE). The TLC plates were developed in TLC chambers with wicks in $CHCl_3$:CH_3OH:H_2O (65/25/4). After development, the TLC plates were air-dried and subjected to autoradiography using Kodak XRP-5 X-Omat film to locate radioactive areas.

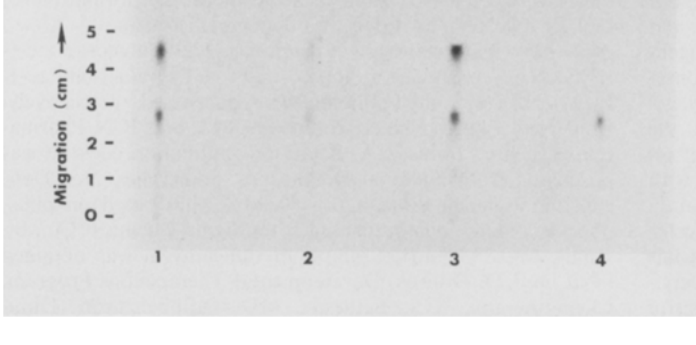
Results and discussion. The effects of DFB and tunicamycin on the formation of GlcNAc- and $(GlcNAc)_2$-pyrophosphoryldolichols are shown in the table. DFB does not appear to affect either the formation of these products or the formation of oligosaccharide lipids or glycoproteins (data not shown). Although these data indicate that DFB does not inhibit GlcNAc or GlcNAc-1-P transferases, the possibility of some other type of membrane effect is not ruled out. Other investigations are underway to determine this.

Conversely, tunicamycin is a potent inhibitor of the formation of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ (see fig.). Inhibition studies with tunicamycin indicated an I_{50} (inhibitor concentration giving 50% inhibition) of 1.5–4 ng/ml, which is slightly less than that reported for enzyme preparations from pig aorta (I_{50} = 10–50 ng)[16].

Lehle and Tanner[17] showed that tunicamycin specifically inhi-

Effects of diflubenzuron and tunicamycin on the formation of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$

	cpm $\bar{X} \pm SD$	Activity (%)
Control	746 ± 101	100
DMSO (2 µl)	783 ± 85	104.9
DFB (48.2 µM)	920 ± 67	123.3
(96.4 µM)	746 ± 129	100
Tunicamycin (10 µg/ml)	205 ± 40	27.4
(20 µg/ml)	174 ± 5	23.3



Autoradiogram of a TLC analysis of the effects of tunicamycin on the formation of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$. *1* Control with Dol·P as the lipid acceptor; *2* tunicamycin (50 ng/ml) with Dol·P as the lipid acceptor; *3* control containing unlabeled Dol·PP-GlcNAc and -$(GlcNAc)_2$ acceptor; *4* same as *3* but with tunicamycin (50 ng/ml) present. Absence of Dol·PP-GlcNAc in *4* indicates it has been converted to Dol·PP-$(GlcNAc)_2$ and that tunicamycin inhibits the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol·P.

bits the first GlcNAc-1-P transfer to Dol·P, but not the transfer of the second GlcNAc residue. This observation has been confirmed in some other organisms, but not in insects. However, several reports have now been made on inhibition of glycoprotein synthesis by tunicamycin in insect tissues, and the authors assumed the mode of action to be interference with the transfer of the first GlcNAc residue to Dol·P[13,14].

Because we and others intend to continue investigating GlcNAc transferases and glycoprotein synthesis in insects, we decided that this mode of action must be confirmed. To determine this, we obtained unlabeled Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ by scaling up the reaction decribed above (ca. 10 times) using unlabeled UDP-GlcNAc. The products were isolated following the procedure described for TLC analysis preparations above. These products were then added to the reaction mixture using [^{14}C]UDP-GlcNAc. If tunicamycin inhibits the first GlcNAc addition to Dol·P then only one labeled product, i.e. [^{14}C]Dol·PP-$(GlcNAc)_2$, should have been observed. This is indeed what happened, as can be seen in the figure. Channel 1 is the normal reaction using Dol·P only as the acceptor, while channel 2 is the same reaction with 50 ng/ml tunicamycin added. Tunicamycin effectively inhibits the transfer of carbohydrate units to Dol·P. Channel 3 is the reaction run with unlabeled Dol·PP-GlcNAc and -$(GlcNAc)_2$. As expected, two radioactive areas corresponding to Dol·PP-GlcNAc and -$(GlcNAc)_2$ are seen in channel 3. When tunicamycin (50 ng/ml) is present in the reaction only the area corresponding to Dol·PP-$(GlcNAc)_2$is observed, indicating that the second GlcNAc addition can proceed but not the first.

1 Mayer, R.T., Chen, A.C., and DeLoach, J.R., Experientia *37* (1981) 337.
2 Deul, D.H., DeJong, B.J., and Vincent, W.R., Pestic. Biochem. Physiol. *8* (1978) 98.
3 Hajjar, N.P., and Casida, J.E., Science *200* (1978) 1499.
4 Hajjar, N.P., and Casida, J.E., Pestic. Biochem. Physiol. *11* (1979) 33.
5 Van Eck, W.H., Insect Biochem. *9* (1979) 295.
6 Cohen, E., and Casida, J.E., Pestic. Biochem. Physiol. *13* (1980) 129.
7 Mayer, R.T., Chen, A.C., and DeLoach, J.R., Insect Biochem. *10* (1980) 549.
8 Mayer, R.T., Meola, S.M., Coppage, D.L., and DeLoach, J.R., J. econ. Ent. *73* (1980) 76.
9 Meola, S.M., and Mayer, R.T., Science *207* (1980) 985.
10 DeLoach, J.R., Meola, S.M., Mayer, R.T., and Thompson, J.M., Pestic. Biochem. Physiol. *15* (1981) 172.
11 Mayer, R.T., Netter, K.J., Leising, H.B., and Schachtschable, D.O., Toxicology, *30* (1984) 1.
12 Quesada-Allue, L.A., Biochem. biophys. Res. Commun. *105* (1982) 312.
13 Miller, S.G., and Silhacek, D.L., Insect Biochem. *12* (1982) 301.
14 Butters, T.D., Hughes, R.C., and Vischer, P., Biochim. biophys. Acta *640* (1981) 672.
15 Mayer, R.T., Meola, S.M., Coppage, D.L., and DeLoach, J.R., J. Insect Physiol. *25* (1979) 667.
16 Elbein, A.D., Gafford, J., and Kang, M.S., Archs Biochem. Biophys. *196* (1979) 311.
17 Lehle, L., and Tanner, W., FEBS Lett. *71* (1976) 167.

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D-ala^2-Metenkephalinamide blocks the synaptically elicited cortical spreading depression in rats

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Summary. Spreading depression (SD) was elicited in rats anesthetized with pentobarbital by a train of 8 electrical pulses (0.1 ms, 10 Hz) applied to parietal cortex. Local application of 50 µg of D-ala^2-metenkephalinamide (DAME) on the stimulated area evoked one or two SD waves followed by an increase of SD threshold from 40 V to 90 V. This effect could be partly prevented by naloxone (1 mg/kg i.p.) and reversed by local application of 4-aminopyridine (10^{-3} M, 2 µl), which reduced SD threshold to 5 and 20 V in normal and DAME-treated cortex, respectively. It is argued that DAME exerts an inhibitory effect on cortical neurons and that the initial SD facilitation is due to initial blockade of inhibitory neurons in the superficial cortical layers.
Key words. Rat cortex; spreading depression; D-ala^2-metenkephalinamide.

Locally applied opioid peptides elicit seizure activity[4,5] and spreading depression[6,7] (SD) in the neocortex and hippocampus, but suppress spontaneous and evoked unit activity when iontophoretically applied into the same regions[8,9]. In an attempt to resolve this apparent contradiction, the effect of a potent enkephalin analogue D-ala^2-metenkephalinamide (DAME) on the threshold of synaptically elicited cortical SD has been examined. SD is a self-propagating neurohumoral phenomenon[10] mediated by accumulation of K^+ ions in the extracellular space to a level causing depolarization of adjacent neurons and release of additional K^+ ions. It is triggered when $(K^+)_e$ exceeds the threshold level of 10–12 mM[11] in a critical volume of cortex (about 1 mm^3)[12]. The $(K^+)_e$ increase can be elicited by sudden activation of the neuronal population through afferent fibers[13]. Assuming that the $(K^+)_e$ threshold and critical volume of SD remain constant, changes of the intensity of electrical stimuli required for eliciting SD reflect the overall excitability of the stimulated region and may serve, therefore as a sensitive index of the drug induced changes of synaptic transmission in the examined network.

Methods. Experiments were performed in 28 male hooded rats of the Druckray strain weighing 200–250 g. The animals were anesthetized with pentobarbital (50 mg/kg) and two adjacent trephine openings 4 mm in diameter were made over the frontoparietal cortex. A pair of spring mounted ball tipped silver wire electrodes with a 2 mm interelectrode distance was placed into the caudal trephine opening. Glass capillary electrodes with a tip diameter of 10–20 µm filled with the physiological saline were introduced 1 mm below the cortical surface, one close to the stimulating electrodes, the other at a point 4–5 mm distant in the rostral trephine opening. The fluid in the capillaries was connected by salt bridges with calomel half cells. Another wick calomel cell electrode applied on the neck muscles served as reference. The potential differences were amplified with high impedance input instrumentation amplifiers and recorded with a conventional polygraph. The electrodes