EFFECT OF CHOLINESTRASE INHIBITORS ON ACETYLCHOLINE AND INSULIN INDUCED GLUCOSE UPTAKE AND CERTAIN HEPATIC ENZYMES IN PIGEON LIVER : AN *IN VITRO* STUDY

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Summary : Insulin and acetylcholine (ACh) are both known to promote glucose uptake by liver of birds. Acetylcholine induced glucose uptake can be predictably potentiated by inhibiting acetylcholinestrase activity. Monocrotophos, acothione (organophosphorus compund) and prostigmine are known inhibitors of acetylcholinestase (AChE). In the present study the action of these three inhibitors of AChE alone as well as in combination with insulin and acetylcholine on *in vitro* glucose uptake by pigeon liver slices was investigated. Both organophorus compounds potentiated the action of insulin as well as acetycholine mediated glucose uptake by liver slices while prostigmine had inhibitory influence. The three compounds also induced alterations in enzyme activities in the liver slices. These results are discussed in detail in the text.

Key words : acetylcholine, acothione, glucose, insulin, Krebs ringer solution, monocrotophos, prostigmine

INTRODUCTION

Promotion of hepatic glucose uptake and glycogen deposition are the mechanisms involved in the hypoglycaemic action of insulin. Apart from insulin, parasympathetic neural involvement too in hapatic glucose uptake and glycogen deposition by way of acetylcholine release has been recognised (19). Owing to the sluggish rate of insulin release in response to glucose loading as well as predominantly glucagon oriented metabolic adaptation in birds (12) acetylcholine mediated glucose transport has assumed greater significance. In a previous *in vitro* study, Pilo and Patel (19) has demonstrated

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increased glucose uptake by pigeon liver slices in presence of acetylcholine. Subsequently proceeding on this assumption they had further demonstrated an increased acetylcholinestrase activity in the liver of pigeons after intravenous glucose loading (20). The increased acetylcholinestrase activity obtained by the above workers in the liver sinusoids after glucose loading is predictably indicative of increased acetylcholine release in the light of the fact proportionate increase in acetylcholinestrase activity occurs immediately subsequent to an increased acetylcholine release. It is safe to assume that acetylcholinestrase activity will always parallel that of acetylcholine release on a temporal scale and hence indirectly could indicate the acetylcholine release on a quantitative level. Pertinently, inhibition of acetylcholinestrase activity could prolong the action of acetylcholine thereby sustaining the influence of acetylcholine on hepatic glucose transport.

Monocrotophos and acothione are two organophosphorus pesticides possesing potent inhibitory action on acetylcholinestrase (1, 2, 3, 9, 28, 29, 34). Prostigmine is the classical and specific inhibitor of acetylcholinestrase activity (17). In the present study all these three compounds have been employed with the primary objective of inhibiting acetylcholinestrase activity. Each of these three inhibitors has been tested individually for its ability to influence acetylcholine or insulin induced glucose uptake process by pigeon liver slices incubated in Krebs Ringer Bicarbonate (KRB) medium. Since it has been reported that the organophosphorus compounds have stimulatory actions on lysosomal enzymes and inhibitory influences on oxidative enzymes such as SDH (13), the quantitative alterations in the activity levels of phosphotases, LDH, SDH, ATPase and phosphorylase in the liver slices have also been assessed in the present study.

MATERIAL AND METHODS

Adult pigeon, *columba livia* weighing 180-200 grams and maintained under laboratory conditions on a diet of grains and water *ad libitum* were used for the experiments. Prior to experimentation, the birds were starved overnight. On the day of experimentation the pigeons were sacrificed by decapitation and the liver rinsed with Krebs ringer medium and then quickly excised. The liver was placed on ice and cut into slices with the blade (100-200 U thickness), weighed and placed in twelve 10 *ml* flasks containing 5 *ml* of Krebs ringer medium. All the twelve flasks contained in addition D-glucose (3 *mg/ml*) and albumin (2 *mg/ml*). They were then divided into subsets of three each of monocrotophos, acothione and prostigmine as additive in the medium. The three flasks of monocrotophos subset contained 1 percent monocrotophos (0.1 *ml*), 1 percent monocrotophos (0.1 *ml*) + insulin (1 *unit/ml*) and 1 percent monocrotophos (0.1 *ml*) + ACh (15 *mg/ml*) while the other two subsets contained 1 percent acothione

(0.1 *ml*) and prostigmine (0.02 *mg/ml*) instead of monocrotophos respectively. Of the remaining three flasks one contained only the basal medium without any additives while the other two contained insulin and acetylcholine respectively as the lone additives. The slices in the flasks were incubated for 90 min at 37°C in a water bath shaker with 120 oscillations/min. After incubation the slices were quickly washed with chilled Krebs ringer buffer and were then processed for the estimation of the glycogen by the method of Seifter *et al.* (25), phosphorylase by the method of Cori *et al.* (6) as adapted by Cahill *et al.* (5), Na^{+}-K^{+}-ATPase by the method of Stanstny (31), acid and alkaline phosphotases by the method described in Sigma Technical Bulletin No. 104 (27), LDH by the method of Varley (33) and SDH by the method of Kun and Abood (18). The values obtained were compared with those values of slices which were not incubated. The measure of glucose uptake or release by the tissue slices were obtained by estimating the glucose content of media before and after incubation as per the micro method of Folin-Malmros (10). In order to confirm the validity of the experimental results, all the experiments have been done in triplicate of 5 samples each and the data statistically analysed for significance by applying Students "t" test.

RESULTS

The data on the effect of cholinestrase inhibitors are presented in Tables I-V which represent the mean values of 5 samples each.

TABLE I : Effect of insulin and acetylcholine on glucose uptake and release by pigeon liver slices. (Mean SEM; N=5).

Additives	*Glucose (mg/100 mg tissue)*		*Glycogen depletion (2)*
	Uptake (1)	*Release (1)*	
None	—	0.8045 ±0.2840	1.5560 ±0.2601
Insulin	1.1446 ±0.2189	—	0.7390 ±0.0364
Acetylcholine	0.5772 ±0.1025	—	1.2789 ±0.1274

(1) Difference between the glucose concentration in the medium before and after the incubation. Mg glucose/100 *mg* liver tissue.

(2) Difference between glycogen values of fresh liver slices and liver slices after incubation. Mg glycogen/100 *mg* liver tissue.

When liver slices were incubated with only glucose as additive in the medium, no uptake of glucose by the slices were observed, instead a release took place. When the medium contained insulin or acetylcholine as additive, glucose uptake took place.

Monocrotophos alone in the incubation medium resulted in an uptake of glucose. Monocrotophos with insulin or acetylcholine had the same effect. However, with acetylcholine glucose uptake was much more than insulin. Acetylcholinestrase activity showed a decrease when monocrotophos was in the medium whether alone or in combination with insulin or acetylcholine. A general decrease of ATPase, alkaline phosphatase, SDH and LDH activities was also observed when liver slices were incubated with mono-

TABLE II : Effect of cholinestrase inhibitors monocrotophos, acothione and prostigmine alone or in combination with insulin or acetylcholine on the uptake or release of glucose by pigeon liver slices under *in vitro* conditions. (Mean SEM, N=5).

Additives	*Glucose*		*Glycogen depletion*
	Uptake(1)	*Release(1)*	
Monocrotophos	0.3433 ±0.0242	—	0.9726* ±0.4373
Monocrotophos + Insulin	0.3592 ±0.0118	—	1.7549+ ±0.4527
Monocrotophos + ACh	0.5186* ±0.0068	—	1.8015* ±0.5698
Acothione	0.5593 ±0.0276	—	1.0984 ±0.1696
Acothione + Insulin	0.6844 ±0.0510	—	1.2151 ±0.2626
Acothione +ACh	0.9553* ±0.0300	—	0.7222 ±0.1208
Prostigmine	0.1869 ±0.0046	—	0.9929 ±0.2026
Prostigmine + Insulin	0.1088 ±0.0045	—	1.2556 ±0.3101
Prostigmine +ACh	0.2681* ±0.0300	—	1.6640 ±0.3851

(1) Mg glucose taken up or released by 100 *mg* liver tissue.
(2) Mg glycogen depletion/100 *mg* liver tissue.
*P<0.05.

and acid phosphatase showed an increase in their activities when acothione was present in the medium whether alone or in combination with insulin or acetylcholine. ATPase, alkaline phosphatase, SDH and LDH all showed a decrease (as was the case with monocrotophos) irrespective of the fact that acothione was present alone or in combination with insulin or acetycholine. Acothione had profound influence over acetylcholinestrase as was the evident from the observation that acetylcholinestrase was inhibited even in the presence of acetylcholine or insulin in the medium.

TABLE IV : Effect of acothione, alone or in combination with insulin or acetycholine on the enzyme activities in the pigeon liver slices under *in vitro* conditions. (Mean ± SEM) N=5.

Enzymes	*Control*(1) *(Tissue)*	*Acothione*	*Acothione+ Insulin*	*Acothione+ ACh*
Na^+-K^+-ATPase Ug phosphorus released/*mg* protein/10 min	29.6305 ±0.4778	16.8363** ±1.0770	21.2622** ±1.0733	22.6576** ±1.2960
AChE Um ACh hydrolysed/mg protein/10 min.	0.7401 ±0.0494	0.3792* ±0.0337	0.4171* ±0.0513	0.2549** ±0.0258
Acid Phosphatase Um P-nitrophenol released/100 *mg* protein/30 min.	156.2681 ±10.5748	246.6196** ±28.7989	238.6776* ±8.4513	203.2963** ±30.2989
Alkaline phosphatase Um p-nitrophenol released/100 *mg* protein/30 min.	23.4093 ±2.0944	20.2047 ±1.7339	20.9121 ±1.8784	22.2488 ±1.2680
LDH Um lactate oxidised/*mg* protein/15 min.	45 4365 ±3.8131	24.8623* ±1.6928	2ọ;9403* ±1.9147	26.8567* ±1.2517
SDH Ug formazan formed/*mg* protein/30 min.	9.2330 ±0.5022	6.3551*** ±0.2389	7.7892*** ±0.3593	7.8580 ±o.4168
Phosphorylase Ug phosphorus released/*mg* protein/10 min.	79.8809 ±4.1955	94.0992** ±3.7417	128.0094** ±10.6409	97.6543* ±7.9786

(1) Enzyme values in the fresh liver slices not subjected to incubation.

*P∠0.05, **P∠0.02, ***P∠0.01

Prostigmine also induced glucose uptake although not as much as that observed with organophosphorus compounds. Prostigmine however, was as effective as monocrotophos and acothione in decreasing the activity of acetylcholinestrase in the liver slices, when present alone or in combination with insulin or acetylcholine. Prostigmine decreased the activity of ATPase significantly in the liver slices while alkaline phosphatase showed no significant variation from the normal level. Acid phosphatase showed a slight but significant decrease, which was contrary to what was observed with monocrotophos and acothione. While SDH showed a decrease, LDH and phosphorylase exhibited an increase in their activities.

TABLE V : Effect of prostigmine alone or in combination with insulin or acetylcholine on the enzyme activities in the pigeon liver slices under *in vitro* conditions. (Mean ± S.E.M.) N=5

Enzymes	*Control*(1) (*Tissue*)	*Prostigmine*	*Prostigmine +Insulin*	*Prostigmine +ACh*
Na^+-K^+-ATPase				
Ug phosphorus released/*mg* protein/10 min.	29.9220 ±0.4406	19.5195** ±0.7311	18.5087** ±0.6648	12.9858*** ±0.6786
AChE				
Um ACh released/*mg* protein/10 min,	0.5619 ±0.0337	0.1618** ±0.0105	0.1994* ±0.0207	0.2597* ±0.0355
Acid phosphatase				
Um P-nitrophenol released/100 *mg* protein/30 mln.	209.9666 ±5.2382	159.3191* ±15.9843	164.9845* ±19.5637	172.7981* ±16.5195
Alkaline phosphatase				
Um P-nitrophenol released/100 *mg* protein/30 min.	19.6661 ±0.8049	19.4880 ±0.6156	21.8463 ±0.7938	20.3450 ±0.3817
LDH				
Um lactate oxidised/*mg* protein/15 min.	53.0170 ±4.0361	95.4141* ±4.2483	84.9537* ±1.2070	89.8445* ±5.4353
SDH				
Ug formazan formed/*mg* protein/30 min.	7.2650 ±0.3620	3.7453* ±0.3736	6.8768 ±0.1883	8.0021 ±0.8595
Phosphorylase				
Ug phosphorus released/*mg* protein/10 min.	145.6178 ±3.7114	169.6982* ±3.8675	152.5810 ±8.2790	185.2083** ±12.6561

(1) Enzymes values in the fresh liver slices not subjected to incubation.

*P∠0.05, **P∠0.02, ***P∠0.01

Glycogen content of liver slices incubated with monocrotophos, acothione or prostigmine, in presence or absence of insulin or acetylcholine, showed a decrease as was observed in all *in vitro* experiments indicating more or less a non-enzymic reduction

Prostigmine with acetylcholine non-significantly increased the SDH activity. Prostigmine with insulin also showed non-significant change in SDH activity. When only prostigmine was an additive in the medium, it significantly reduced the SDH activity.

DISCUSSION

Anticholinestrase compounds bind with anionic or esteric site of the enzyme and interfere with the orderly interaction between enzyme and the substrate (29). A difference in the degree of inhibition of cholinestrase activity in different tissues by organophosphorus compounds has been reported by a number of workers (7, 14, 21, 26). It has also been shown that brain gets least affected compared to liver and muscle due to presence of a blood brain barrier which does not permit the penetration of organophosphorus compounds Both monocrotophos and acothione by inhibiting acetylcholinestrase activity can sustain the action of acetylcholine for longer time and thereby potentiate its influence. In this light it is presumable that inhibition of acetylcholinestrase by monocrotophos, acothione or prostigmine in the present study could lead to a temporarily prolonged action of acetylcholine. Since it has been already established that acetylcholine has an action akin to that of insulin in glucose uptake by hepatocytes (19), it is conceivable that prolongation of acetylcholine action resulting from inhibition of cholinestrase should promote greater glucose uptake. The present result do favour such a conclusion by the observed uptake of glucose in presence of all the three cholinestrase inhibitors when they were present as the only additive in the incubation medium. The favourable influence of the three cholinestrase inhibitors on glucose uptake in this context is clearly manifested by the absence of glucose uptake when the tissue slices were incubated in Krebs media without any of above three inhibitors. In this sense the increased glucose uptake demonstrated by both monocrotophos and acothione in presence of acetylcholine needs no explanation and the increase in glucose uptake obtained was of the order of 51 percent and 65 percent respectively.

However, prostigmine seems to have brought about reduced glucose uptake in presence of acetylcholine than when prostigmine was present alone. The reduction was about 63 percent which is quite substantial. Relevant in this context is the reduced glucose uptake by prostigmine alone as the additive in the medium which was nearly about one third to one fifth of that recorded for monocrotophos and acothione respectively as the alone additive in the medium. Obviously, though prostigmine has as much

greater inhibitory influence on acetylcholinestrase, it nevertheless has much less effeciency in promoting glucose uptake. This enigmatic observation needs alternate explanation and warrants further studies. In contrast none of the three inhibitors had any influence on insulin induced glucose uptake.

The data on the effect of the three cholinestrase inhibitors when present alone or in combination with insulin or acetylcholine showed generalised inhibitory influence on Na^+-K^+-ATPase, acetylcholinestrase, alkaline phosphatases, LDH and SDH while there was a stimulatory influence on acid phosphatase and phosphorylase activities. Though the reduction in the activity of acetylcholinestrase is understandable, the fall in activity levels of LDH and SDH together with that of Na^+-K^+-ATPase may have to be viewed in the context of decreasing metabolic efficiency of the tissue slices. Pertinent to quote in this context are the reports of Joshi (13) who has also shown reduced activity of these enzymes by organophosphorus compounds in her *in vivo* studies on *Tilpia mosambica*. The reduced alkaline phosphatase activity is also indicative of an inhibitory influence on many specialised cell functions which are accredited to alkaline phosphatase (4, 18, 22. 23, 24, 30, 31). The increased acid phosphatase and phosphorylase activities in all the experimental set ups may denote increased lysosomal activity and carbohydrate catabolism which is reflected in the recorded depletion of glycogen content of the liver slices.

In the conclusion, it may be assumed that the organophosphorus compound by their inhibitory actions on acetylcholinestrase favours glucose uptake by acetylcholine on one hand, and brings about decreased metabolic functioning on other hand by their inhibitory influence on various enzyme systems. Though these conclusions are drawn with reference to present *in vitro* studies, similar actions under *in vivo* conditions are also feasible.

REFERENCES

1. Aldridge, W.N. The differentiation of true and pseudocholinestrase by organophosphorus compounds. *Biochem J.*, **53** : 62-67, 1953a.
2. Aldridge, W.N. Organophosphorus compounds and carbamates and their reaction with estrases. *Brit. Med. Bull.*, **25** : 236-240, 1969.
3. Aldridge, W.N. and A.N. Dawson. The inhibition of erythrocyte cholinestrase by tri-esters of phosphoric acid : 1. Diethyl p-nitro phenyl phosphate (E600) and analogues. *Biochem. J.*, **51** : 62-70, 1952.
4. Bradfield, J.R.G. Phosphatases and nucleic acids in silk glands, cytochemical aspects of fibrillar protein secretion. *Quart. J. Micr. Sci.*, **92** : 87-112, 1951.

5. Cahill, G.F. Jr., J. Ashmore, S. Zottu and A.B. Hastings. *J. Biol. Chem.*, **224** : 237-250. Cited from Van de Verve, (1981), 1957.

6. Cori, C.F., C.T. Cori and A. Green. Crystalline muscle phosphorylase, III Kinetics. *J. Biol Chem.*, **151** : 39-55, 1943.

7. Dikshith, T.S.S., K.K. Datta, H.S. Hushwah, P. Chandra and R.B. Raizards. Effect of methyl dimeton on vital organs and cholinestrase in male rats. *Indian. J. Exp. Biol.*, **18** : 163-167, 1980.

8. Fiske, C.H. and Y. Subba Row. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66** : 375-400, 1925.

9. Fleisher, H.J., W.L. Harris and T.P. Berkowitz. Dephosphorylation *in vivo* of brain acetylcholinestrase inhibited by isopropyl methylphosphofluoridate (sarin). *Biochem. Pharmacol.*, **19** : 421-426, 1970.

10. Folin, O. and H. Malmros. Micromethod for glucose estimation in blood. *J. Biol. Chem.*, **83** : 115, 1929.

11. Guenther, V. and S. Klaus. Red cell and plasma cholinestrase activities in micro samples of human and animal blood determined simultaneously by a modified acetylthiocholine/DTNB procedure. *Toxicology. Appl. Pharmacol.*, **16** : 764-772, 1970.

12. Hazelwood, R.L. The avain endocrine pancreas. *Am. Zool.*, **13** : 699-706, 1973.

13. Joshi, U. Effect of monocrotophos on *Tilapia mossambica*. A Ph.D. thesis submitted to the Maharaja Sayajirao University of Baroda, 1982.

14. Koundia, R.P. and R. Ramamurthy. Effect of sumithion (Fenitrothion) on some selected enzyme systems in the fish *Tilapia mossambica* (Peters) *Indian. J. Exp. Biol.*, **16** : 809-811, 1978.

15. Kun, E. and L.G. Abood. Colorimetric estimation of succinic dehydrogenase by triphenyl tetrozolic chloride. *Science*, **108** : 144-146, 1949.

16. Lowry, O.H., N.J. Rosenbrough, L.R. Farr and J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** : 265-275, 1951.

17. Lahue, R. Methods in Neurobiology, edited by : Robert Lahue. *Vol.* **2** : 1981.

18. Moog, F. The physiological significance of the phosphomonoestrases. *Biol. Rev.* **21** : 41-59, 1946.

19. Pilo, B. and P.V. Patel. Influence of insulin and acetylcholine on transport of glucose and glycogen deposition in liver slices of pigeon and rat. *Indian. J. Exp. Biol.*, **16** : 929-932, 1978.

20. Pilo, B. and P.V. Patel. Neuroendocrine control of uptake and metabolism of glucose in avian liver. *Pavo.*, **16** : 162-178, 1978.

21. Quari, S.S.D. and M. Ahmed. Duration of phosphomidon induced changes in cholinestrase activity in different organs of rats. *Indian. J. Exp. Biol.*, **17** : 423-424, 1979.

22. Rackallio, J. Enzyme histochemistry of wound healing frog. *Histochem. Cytochem.*, **1** : 1-101, 1970.

23. Rogers, K.T. Studtes on chick brain of biochemical differentiation related to morphological differentiation and on set on functions. III. Histochemical localization of alkaline phosphatase. *J. Exp. Zool.*, **145** : 49-55, 1960.

24. Rosenthal, I.M., S.L. Bonting, W. Hogan. and C.L. Pirani. *A. M. A. J. Disease children*, **99** : 185-192. Cited by Burston, M.S. 1962 : In "Enz. Histochem. and its applications in the study of neoplasm". pp 164. Acad. Press, New York, 1960.

25. Seifter, S.. S. Dalton, S. Novic and B. Muntwyter. The estimation of glycogen with anthrone reagent. *Arch. Biochem.*, **25** : 191-200, 1950.

26. Sharma, R.P. J.L. Shupe acd J.R. Potter. Tissue cholinestrase inhibition by 2-carbomethoxy-l-methylvinyldimethyl phosphate (Mevinphos). *Toxicol. Appl. Pharmacol.*, **24** : 245-242, 1973.

27. Sigma Technical Bulletin, No. 104, Sigma Chem. Co., 3500 Dekalb St. Louis 18, Mo, USA.

28. Simpson, R.E., G.R. Simpson and D.J. Renney. Poisoning with monocrotophos an organophosphorus pesticide. *Med. J. Aust.*, **2** : 1013-1016, 1969.

29. Silver, Ann. "The biology of cholinestrase". In : Frontiers of Biology. (eds.) A. Neuberger and E.L. Tatum. North-Holland Publishing Co., Amsterdam, Oxford, 1974.

30. Simkiss, K. Phosphataes crystal poisons of calcification. *Biol. Rev.*, **39** : 487-503, 1964.

31. Sols, A. Fosfatases. Revision de la literature de 1947-1948. *Resp. Fisiol.*, **5** : 57-129, 1947.

32. Stastny, F. Hydrocortisone as a possible inducer of $Na^+ - K^+ -$ ATPase in the chick embryo cerebral hemisphere. *Brain Res.*, **25** : 397-410, 1971.

33. Varley, H. 1975. Colorimetric method. In : "Practical clinical Biochemistry", by Varley, H. Published by Arnold Heinemann Publishers (India) Private Ltd., Indian Edition, 1975.

34. Verberk, M.M. Incipient cholinestrase inhibition in volunteers ingesting monocrotophos as mevinphos for one month. *Toxicol. Appl. Pharmacol.*, **42** : 345-350, 1977.