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FINAL REPORT

PROJECT No. I-56-79

**Biochemical Aspects of the Effect of
Altered Compositions of Atmospheric
Gases on Stored Product Insects and the
Role of Carbon Dioxide in Enhancing the
Activity of Some Conventional
Insecticides**

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ABSTRACT

Research has been done on the biochemical effects of carbon dioxide on the pentose phosphate shunt, the "malic" enzyme, the sorbitol path and the glutathione status in Ephesia cautella (Wlk) pupae.

In addition research has been extended to larvae of E. cautella, as well as to the closely related Plodis interpunctella, with the aim of extending our studies to the mitochondrial enzymes. It was found that both species responded to CO₂ in a similar fashion. Also the correlation between juvenile hormone (JH) titer and CO₂ susceptibility was examined after it was found that JH titer reaches a minimum at about the same age CO₂ susceptibility was lost. By starving larvae a physiological state was created in which respiration was minimal and in which growth and development were not occurring.

It was found that 6-phosphogluconate dehydrogenase, a key enzyme in pentosephosphate shunt, was inhibited by over 10% CO₂. "Malic" enzyme is inhibited 12% at 65% CO₂ in the oxidative direction and 45% in the reductive direction at the same level. The system becomes completely thermodynamically controlled at that level, thus favoring the transformation of malate to lactate; thus two pathways producing NADPH are markedly blocked.

The sorbitol pathway is activated under hypercarbia reaching a maximum production of sorbitol at 80% CO₂.

Glutathione (GSH) is reduced significantly to a fixed level by treatment with 90% CO₂ for 24 h and longer. If treated 24 h, the GSH level returns to the control value after 72 h. Although the synergism of methyl bromide by CO₂ is well documented, the pretreatment of pupae with CO₂, before methyl bromide fumigation does not decrease the GSH level significantly with respect to methyl bromide treatment alone.

It was found that CO₂ effectiveness in killing both Ephestia and Plodia larvae depended upon the length of exposure and the age of the larva, young larvae being more susceptible to the shorter exposure than mature larvae.

Loss of mitochondrial content correlates well with the observed loss in sensitivity to CO₂ treatment.

Decreased oxidative capacity in the mitochondria (malate-pyruvate oxidation) also contributes to the decline in live respiration. On the other hand, succinate oxidative activity increases throughout the last instar until larvae reach the wandering stage; this would not support the proposition that succinic dehydrogenase inhibition is the basis for CO₂ toxicity. Our data seem to indicate that susceptibility to CO₂ treatments seem to rely on the total mitochondrial oxidative capacity of the insect.

INTRODUCTION

Losses of stored grain caused by storage pests amount to about 3.9 percent in the U.S. and in Israel, a grain importing country, to about 1% (excluding the cost of control). The chemical methods used for pest control are often a hazard to personnel applying them, and, mainly by leaving toxic residues, a health risk to the consumer.

In addition to the residue problem, the development of insect resistance (Champ and Dyte, 1976) is a matter of grave concern for those planning the control strategy for insect pests.

An alternative technique available for insect control in post-harvest situations is the application of modified atmospheres. This technique basically involves changing the proportions of the normal constituents of the storage environment O₂, N₂ and CO₂ to create an atmosphere lethal to insects. One of the first applications of carbon dioxide to a silo

has been described by Jay et al. (1970).

Although the method is regarded as "non-chemical," it was soon evident that carbon dioxide had significant biochemical effects on the insect mechanism (Navarro and Friedlander, 1975; Friedlander and Navarro, 1979). The aim of this project was to investigate these and possibly use them for increasing the efficiency of the method.

Carbon dioxide taken in absorbed or dissolved form through the stomach is considered safe, so its use as a "pesticide" would present no toxicological problems. Its respiratory toxicity is species- and strain-dependent. Humans lose consciousness in air containing 10% CO₂ and more (Merck Index, 1976). While cats succumb after 5 min of 64% CO₂, the mean survival time of wild rabbits at 50% CO₂ is 71 minutes and of domestic rabbits 23 min (Hayward and Lisson, 1978). Guinea pigs were found to be much more susceptible to CO₂ than rats (Schaefer et al., 1973). On the basis of their findings and the literature they quoted, Hayward and Lisson (1978) came to the conclusion that generally fossorial mammals can adapt physiologically to increased CO₂ concentrations. It is therefore to be expected that insects, especially stored product pests who live inside heaps of grain or similar commodities are much more resistant to the gas.

Navarro and Calderon (1974) found that 21% CO₂ failed to cause, at high relative humidity, complete mortality of E. cautella pupae even after 6 days' exposure.

Small increases in carbon dioxide reduced somewhat the longevity and the rate of increase of Sitophilus zeamais (Spratt, 1974). Mortality can be increased by decreasing relative humidity (Jay et al., 1971; Navarro and Calderon, 1974), this being accompanied by desiccation of insects (Jay et al., 1971).

A number of physical and physiological effects have been noted for a long time, such as the opening of the spiracles (Hoyle, 1960), effects

on vitellogenin metabolism (Engels et al., 1976), initiation of oogenesis (Engels and Ramamurthy, 1976), growth retarding and mating efficiency (Brooks, 1957; Lum, 1974) and others. (Fuseau-Braesch and Nicolas, 1972). From the point of view of pest control, the synergistic effect with a number of fumigants and pesticides is, perhaps, one of the most interesting. Cotton and Young observed as early as 1929 that CO₂ increased the efficacy of fumigants. Jones (1938) presented evidence that the increased toxicity of fumigant-CO₂ mixtures is not simply due to mechanical causes such as prolonged opening of the spiracles, but apparently dependent on physico-chemical factors at the molecular level.

Up to recent times, very little research had been done on the biochemical aspects of the problem. The physiology of carbon dioxide transport in insect blood was investigated by Levenbook (1950); Edwards (1968) and Edwards and Batten (1973) correlated CO₂-anesthesia with the inhibition of succinic dehydrogenase but came to the conclusion that this was not the only factor involved.

In our first investigations we found that increasing CO₂ levels caused a steady increase in hemolymph lactate; oxygen decrease did not have a similar effect except at very low levels (3% or less) in E. cautella pupae (Navarro and Friedlander, 1975).

Previous investigations in our laboratory (Navarro and Friedlander, 1975; Friedlander and Navarro, 1979) have shown that CO₂ treatment caused, in addition to a drastic increase in lactate and alanine in Ephestia cautella pupae, a reduction of adenosine triphosphate, thus causing a significant drop in the energy charge of the system. This would point to a lesion of the respiratory cycle (Wyatt, 1963), and might explain some of the phenomena noticed, such as a retardation of development (delay of eclosion of the pupae) (Atkinson, 1968), but not, for instance,

the synergistic effect of CO₂ in conjunction with some conventional fumigants and pesticides. Also the fact that citrate, malate and glutamate did not accumulate in treated insects, made us assume that additional biochemical systems were attacked. We therefore looked at the pentose phosphate cycle (Lehninger, 1975), the "malic" enzyme (E.C.1.1.1.40) both suppliers of NADPH necessary for the functioning of mixed function oxidase (Kornberg, 1976; Thurman et al., 1975), the sorbitol pathway (Friedlander and Navarro, 1983) and the glutathione conjugation system (Boyland and Chasseaud, 1969). The two latter systems are both involved in the detoxification of many insecticides. The rate of mixed function oxidase catalyzed detoxifications depends on the NADPH level (Thurman et al., 1975). Methyl bromide and similar fumigants are detoxified by conjugation with glutathione; their action is synergized by carbon dioxide (Bond and Buckland, 1978).

Further points of interest were the interaction of carbon dioxide and the juvenile hormones and their synthetic analogs as well as the change of susceptibility with age.

Line of Research and Methods

Biochemistry

Pentosephosphate shunt

The activities of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase in homogenate of Ephesia cautella pupae, 0-24-h-old, were determined by the methods listed in Table 1. Activities of 6-phosphodehydrogenase were also determined in the presence of bicarbonate and of dissolved CO₂ (Bendall et al., 1958).

Ribulose-5-phosphate was determined in young untreated as well as in pupae, 0-24-h-old, treated with atmospheres of different compositions.

Sorbitol pathway

The activities of the key enzymes and of glucose, fructose and sorbitol

were determined as described (Friedlander and Navarro, 1983) in untreated pupae exposed to various controlled atmospheres.

Controlled atmospheres and glutathione

Glutathione levels were determined in pupae exposed to atmospheres of various compositions according to the method of Starratt and Bond (1981) with slight modifications.

In some experiments, pupae exposed to hypercarbic atmospheres were also exposed part of the time, to methyl bromide, to determine differences in glutathione levels.

Glutathione disulfide was determined with glutathione reductase (Klotzsch and Bergmeyer, 1965).

Physiology

Bioassays were used to test the combined effects of gaseous CO₂ and the insect growth regulator Altosid, by exposing larvae of E. cautella that had previously received 5 ppm Altosid in their diet to atmospheres containing 35% and 20% CO₂ in air; mortality and pupation percentages were determined.

Bioassays were also used to determine the combined effect of starvation and carbon dioxide on the test insect. Details will be given under "Results."

Respiration, in some instances, was measured with a Warburg Manometer and the respiratory quotient determined.

Ephestia cautella (Wlk) pupae were used in the Bet Dagan laboratory; this insect has been thoroughly investigated in Israel. In Gainesville, Plodia interpunctella is more standardized and available and, furthermore, has been found to respond similarly in the initial studies to Ephestia. Therefore, the cooperating scientist has concentrated in his studies on Plodia.

RESULTS

Biochemistry

Pentosephosphate shunt

Table 2 gives the ribulose-5-phosphate levels in untreated and treated pupae, after 24 h and 48 h treatment. Table 3 gives the influence of CO₂ on the activity of 6-phosphogluconate dehydrogenase in the absence and presence of carbon dioxide. In freshly prepared homogenate the initial activities were

Glucose-6-phosphate dehydrogenase 47.2±5.1 nanomoles/min/mg protein

6-Phosphogluconate dehydrogenase 15.3±1.8 nanomoles/min/mg protein

"Malic" enzyme (EC 1.1.1.40)

In pupal homogenates, activity of this enzyme was found to increase from pH 6.5 (zero activity) to pH 8.4 (maximal activity). Results of measurements of the decarboxylation rate of malic acid or the carboxylation rate of pyruvic acid at pH 7.4 are given in Table 4.

Sorbitol pathway

For levels of glucose, fructose and sorbitol, see Friedlander and Navarro (1983; attached). A second table in that publication gives the influence of bicarbonate on aldose reductase.

Sorbitol increases to a maximum of 80% CO₂, and then decreases. The reduction of D-glyceraldehyde is reduced to 67% by 0.1% bicarbonate in the reaction medium. The isomeric dihydroxyacetone, a less good substrate, is affected less. Glucose, the poorest substrate for this enzyme is not affected at all by bicarbonate addition.

Inspection of the kinetic constants (Table 7) shows that only K_m (glyceraldehyde) is affected, thus indicating competitive inhibition.

Glutathione

Table 5 shows the glutathione levels of untreated pupae as well as

of pupae treated for 24 h in atmospheres of various compositions. Table 6 gives the recuperation levels of pupae treated for 24 h in an atmosphere of 90% CO₂, after terminating the exposure; the glutathione continues to fall for at least 48 hours, after 72 hours the pretreatment level of 1.68 μ mole/g is recovered.

Treatment of pupae with methyl bromide caused a drastic reduction of glutathione as expected (Starratt and Bond, 1981); pretreatment of the pupae by 90% CO₂ for 24 h did not change this reduction in any way (Table 7).

Only traces of oxidised glutathione were found in all samples examined.

Physiology

Delay of development and mortality

Exposing Ephestia cautella pupae, 0-24-h or 24-48-h old to 90% CO₂ for 24 h delays eclosion by 3 days without increasing mortality or changing ovipository capacity.

Newly molted last instar larvae died within 4 days when the atmosphere contained 30 or 35% CO₂ in the air.

Effect of carbon dioxide on mitochondrial enzymes of Plodia interpunctella larvae

Previous studies on pupae had suggested an impairment in the metabolism of carbohydrates. We wanted to extend those studies to the mitochondrial enzymes, since they catalyze the final combustion of ingested foodstuffs to CO₂ and H₂O. The low quantities of mitochondrial enzymes during metamorphosis prompted us to examine these activities in last instar larvae. Our initial studies examined larvae of Ephestia cautella and larvae of the closely related species, Plodia interpunctella. Both moth species responded similarly in the initial studies, so more detailed experiments on the mechanism of CO₂ lethality were limited to Plodia which is a more standardized and available insect in the laboratory in Gainesville.

In all experiments we used the previously selected 80% CO₂:20% O₂ gas mixture as the test atmosphere. We found that the effectiveness of this gas mixture in killing moth larvae depended upon the length of the exposure and the age of the larva. Typically, young larvae were found more susceptible to the shorter exposures of CO₂ than were mature larvae (Fig. 1). Wandering stage larvae were found to recover from exposures of as long as 24 hours. We concluded that an exposure between 12 and 24 hours would provide a suitable exposure for our experiments comparing the effects of CO₂ treatment on the metabolic state. For convenience, an overnight treatment of 16 hours was selected as the standard exposure for the experiments reported here.

We also observed in these experiments that younger larvae were more easily killed than older larvae. Examining this more closely, we found that about midway through the last larval feeding period, susceptibility to CO₂ treatment dropped dramatically. A comparison of Ephestia with Plodia indicated that both species responded to CO₂ treatment in similar fashion, showing a marked reduction in mortality after achieving a body weight of 8 mg (Fig. 2). These data describe our biological test system in which larvae abruptly change from susceptible to CO₂ poisoning to non-susceptible even though feeding and growth continue and hormonal secretion changes are not evident.

However, previous studies on Plodia indicated that juvenile hormone (JH) titer reaches a minimum level in the larvae at about the same age CO₂ susceptibility is lost. We hypothesized that if decreased JH titer decreases CO₂ susceptibility, then administration of exogenous JH should prolong the period of susceptibility to CO₂ poisoning. We tested this hypothesis in both Plodia and Ephestia and found a minimal but definite effect of JH on CO₂ toxicity (Fig. 2). In fact, after CO₂ treatment, surviving larvae continued to grow into giant larvae, characteristic of JH

ingestion at the same rate as untreated larvae. Concurrent with the mortality studies, respiration of Plodia larvae reared in the presence and absence of JH was measured with a Warburg manometer. Newly molted (3-5 mg) larvae feeding on JH diet respired at a rate 50% higher than untreated larvae of the same age (Fig. 3). As larvae grew, this respiratory difference diminished. Measurement of the respiratory quotient (RQ) indicated that carbohydrates were being utilized ($RQ = 1.0$) at all ages during the last larval instar. Treatment of larvae with JH did not alter these RQ values.

In determining the RQs, we observed that the accumulation of expired CO_2 in the Warburg flasks stimulated the respiration of the test insects. Most notable was a two-fold stimulation in 3-5 mg larvae; CO_2 stimulation was appreciably reduced in older larvae (Fig. 3). Maximum CO_2 concentrations achieved never exceeded 1% of the atmosphere during the Warburg run.

The data summarized in Figs. 2 and 3 indicated that the greatest susceptibility to CO_2 poisoning in Plodia occurred during the developmental period when respiration was most intense. Therefore, we planned a series of experiments using 6-8 mg Plodia larvae in which respiration could be experimentally shut down without initiating metamorphosis. Fig. 4 summarizes growth data which demonstrates that starvation leads to an initial weight loss, followed by a much diminished weight loss, culminating in death after 8 days of starvation. We examined respiration during the first 48 hours of starvation (Table 8) and found that O_2 consumption decreased to one-half within the first 16 hours of starvation and remained low for the remainder of the starvation period. Thus, we were able to create a physiological state in which respiration was minimal and in which growth and development was not occurring.

We subsequently tested the effects of a 16-hour exposure to an atmosphere of 80% CO_2 :20% O_2 after varying intervals of starvation. Starvation

throughout the test period resulted in 83% mortality after 8 days; exposure to CO_2 for 16 hours with no starvation resulted in 100% mortality within 4 days (Table 9). Starvation for 12 or 24 hours followed by a 16-hour CO_2 treatment also resulted in high mortality. However, mortality was considerably reduced when a 36-hour starvation period preceded the CO_2 treatment. Starvation for 12, 24, 36, 28, 40 and 52 hours was followed by a resumption of feeding growth and development when the larvae were returned to diet. These data indicated that the effects of starvation for a period as long as 52 hours was reversible upon refeeding; this could be prevented by a CO_2 exposure, particularly if starvation has not progressed beyond 24 hours. We interpreted these observations as indicating that the initial metabolic shutdown (as indicated by decreased O_2 consumption) was a controlled response to the loss of oxidizable foodstuffs; longer shutdown resulted in the loss of the oxidative machinery within the larval tissues as well.

To test our idea, we focused our study on the mitochondria because within them resides the oxidative enzymes responsible for converting ingested food into utilizable energy. We first determined the mitochondrial content in larvae of different ages. It is evident from the data in Table 10, that mitochondrial content declines rapidly after larvae reach a weight of 8 mg, decreasing to almost half by the wandering stage. The loss of mitochondria correlates well with the observed loss in sensitivity to CO_2 treatment (Fig. 2). We have not yet examined the effects of starvation on mitochondrial content. It is tempting to predict that longer term starvation would lead to mitochondrial loss concurrent with the decreased sensitivity to CO_2 noted previously.

In additional experiments on developing larvae, we found that decreased oxidative capacity in the mitochondria (pyruvate-malate oxidation) also contributes to the decline in live respiration (Table 10). Pyruvate-malate

oxidation was chosen as an indicator because of its central role in the combustion of food in the insect. Another mitochondrial enzyme, succinic dehydrogenase, was examined because of its sensitivity to CO₂ inhibition. The data shown in Table 3 reveal that succinate oxidative activity increases throughout the last instar until larvae reach the wandering stage. These data would not support the proposition that succinic dehydrogenase inhibition is the basis for CO₂ toxicity in Ploedia larvae.

The data reported in this study indicate a mitochondrial role in CO₂ toxicity. Susceptibility to CO₂ treatments seem to rely on the total mitochondrial oxidative capacity of the insect. Shutting down mitochondrial enzyme activity by short-term starvation did not in itself decrease susceptibility. It was only when the amount of mitochondria decreased per unit body weight that we saw a decrease in susceptibility. Therefore, susceptibility appears to be correlated with the number of mitochondrial oxidative units per gram tissue as well as the oxidative capacity of the hexose monophosphate shunt reported previously.

DISCUSSION

The reduction of the ATP level by hypercarbia seems to be a major lesion (Friedlander and Navarro, 1979); the activation of the sorbitol pathway (Friedlander and Navarro, 1983) only stresses this point (Chino, 1963). Other systems affected are the malate → pyruvate oxidation and the pentose phosphate shunt, suppliers of NADPH needed for biosynthetic reactions and for mixed function oxidase catalyzed detoxifications. It may be assumed, under hypercarbic conditions, by comparing the analytic and kinetic data, that these reactions depend largely on the availability of malate. Edwards (1968) believed at first that the inhibition of succinic dehydrogenase by CO₂ was the cause for the "knock-down" of the

corn earworm. Our findings show that this assumption is unnecessary: by drastic reduction of malate and of oxaloacetate with which malate is in equilibrium, the Krebs cycle and respiration will be slowed down. It might be argued that the reduction of malate levels are precisely caused by the blocking of succinate dehydrogenase. The synthesis of malate from pyruvate, especially the presence of an excess of carbon dioxide, is thermodynamically favored over the reverse reaction. However, by coupling the oxidative reaction to the reduction of pyruvate to lactate, the malate $\rightarrow$ pyruvate path becomes much more favorable, especially as the reversibility, as shown by the reaction rates, becomes perfect under conditions of hypercarbia.

It is not yet possible to obtain a clear picture of glutathione and hypercarbia. Exposure of 0-24-h old pupae to 90% CO_2 causes a fall of glutathione, to a value that remains constant even if hypercarbia is continued. If the insect is returned to a normal atmosphere, the glutathione returns to the normal level after 72 h. Although CO_2 synergises the fumigant methyl bromide, which is detoxified by conjugation with glutathione (Starratt and Bond, 1981), there is no difference in glutathione levels between pupae exposed to methyl bromide alone and those exposed to methyl bromide plus carbon dioxide. Since only traces of glutathione disulfide could be detected in both treated and untreated insects, the effect of carbon dioxide could be on the hydrolysis of pyroglutamic acid, a stage of glutathione biosynthesis requiring ATP (Meister and Tate, 1976), or on the release of glutathione from glutathione-protein complexes (Kosower and Kosower, 1978).

A physiological effect, deserving attention is the delay of development in pupae treated for 24 h with 90% CO_2 .

As early as 1942, Oosthuizen & Schmidt noticed that the larval development of the cowpea weevil was retarded by a low concentration and long

exposure to carbon dioxide treatment. Other examples of growth retardation have been reported later, e.g., of the German cockroach (Brooks, 1957), the corn earworm (Edwards, 1968), and others.

The effect of carbon dioxide on larval duration and adult body size has been dealt with thoroughly by Tanaka (1982), who did not add any further evidence concerning the mechanism of action of carbon dioxide to what had already been surmised by Edwards (1968) and Brooks (1965), but thought the gas to be an interesting tool to be used to affect the developmental processes of insects. We believe that the reduction of the energy charge by CO₂ treatment (Friedlander and Navarro, 1979), which implies an inhibition in biosynthesis of proteins (requiring nucleotide phosphates), can be used to rationalize the delay in development (Atkinson, 1968). However, this is probably not the only effect of CO₂ since a number of physiological effects, for instance, the effect on vitellogenin metabolism in queen honeybees (Engels et al., 1976), cannot be easily explained that way. At any rate, our explanations cover a large part of the phenomena involved in storage pest control by CO₂.

Summing up, we can say that carbon dioxide affects the following:

- (a) the respiratory enzymes; this fits very well with the finding of our American colleague that loss of mitochondrial content correlates with loss in sensitivity to CO₂ treatment. Reduction of nucleoside phosphates and the energy charge would also explain the delay in development of pupae.
- (b) the pentose phosphate shunt.
- (c) the "malic" enzyme catalyzing the transformation of malate to pyruvate and vice versa. The cooperating scientist found (p.) (Fig. 3) that in developing 5th instar larvae, pyruvate-malate oxidation activity increased up to the developmental stage, corresponding to a live weight of 6-8mg, in parallel to the increase of live respiration. The arguments

presented by the cooperating scientist show definitely that succinic dehydrogenase inhibition is not the basis of carbon dioxide toxicity.

(d) system or systems involve glutathione. Carbon dioxide is definitely involved as an inhibitor either in the production or in the regeneration of glutathione. At this stage it is not possible to pinpoint the effect.

Cooperation

Reference has been made in the above report, about the various immediate results obtained by cooperation. By using a more physiological approach, Dr. Silhacek was able to bring out a number of points in support of our findings based mainly on biochemical reasoning. Thus Dr. Silhacek's finding that the increase in importance of the "malic" enzyme up to a live weight of 6-8 mg, and our finding that this enzyme is inhibited by CO₂ might indicate a reduction in mitochondrial function, thus diminishing the activity of respiratory enzymes.

Indeed, it was essentially Dr. Silhacek who found a connection between mitochondrial content and susceptibility to CO₂. It is still necessary to collect, compare and assess results in both laboratories. It can, however, be said that we have come to a more thorough understanding of the biochemical and physiological action of carbon dioxide, in which the Gainesville laboratory has been fully exploited.

Conclusive Remarks

We have shown that, although the use of modified atmospheres for pest control is classified as a "non-chemical" method, the biochemical and toxicological effects of the gas are of extreme importance.

A major advantage of the use of carbon dioxide as an insecticide is that a direct residue problem does not exist. There are, however, reports

that CO₂ causes the secretion of p-benzoquinones in Tribolium castaneum (Irwin et al., 1972). These compounds are potential carcinogens and the problem definitely deserves an investigation. It is, however, beyond the scope of our work.

In addition to its own toxicity to insects, which has been amply investigated and reported (Jay et al., 1970, 1971), two points are of interest in pest control, namely, synergism and resistance. We have referred in greater detail to synergism in a previous section of this report. Resistance to carbon dioxide has been reported first by Bond and Buckland (1979), who claimed a 3-fold increase in CO₂ tolerance in Sitophilus granarius adults after giving CO₂ treatment successfully to seven generations.

It is hard to visualize the resistance to carbon dioxide as a biochemical detoxification mechanism of the kind we are used to see in the detoxification of, say, malathion. Unless the basis of the resistance is morphological and not biochemical, its mechanism is of an unknown kind. Thus, many problems remain waiting for a solution. We claim, however, to have found tools to be used in further investigations. An important tool is the adenylate energy charge (Atkinson, 1968).

It has been postulated that ATP-requiring reactions are activated by a high energy charge, while ATP-producing reactions proceed fastest at a low energy charge. Thus, by reducing the energy charge, CO₂ could be the basic cause for a number of vital processes. Growth depends essentially on protein synthesis which is certainly ATP-dependent, or depending on the nucleotide triphosphates whose level ultimately depends on ATP. Nervous impulses depend on the acetylation of choline and deacetylation of acetylcholine. According to Nachmansohn (1957) the energy for this process is derived from ATP. Indeed, Lavon and Agrest (1968) found an inhibition of the acetylation of choline in rats with

chronic hypercapnia; they believe it was caused by a decrease in ATP. ATP or the energy charge thus have a definite neurochemical function, through which CO_2 can act.

Earlier in the text we pointed out the connection between inhibition of NADPH producing pathways and the functioning of mixed function oxidases (Thurman et al., 1975) and the bearing of this on synergism of pesticides by CO_2 . The interaction of carbon dioxide with the glutathione is not so clear and we hope to investigate it further.

Benefit to Agriculture

The importance of grain storage under modified atmospheres, avoiding the use of toxic pesticides cannot be stressed enough. However, like every other pesticide, it cannot be used properly before its action on the insect organism is known. Strangely enough, until recently very little was known on the toxicology of carbon dioxide. We hope that our work will make some contribution towards better understanding of this subject and, as such, help to use carbon dioxide more efficiently.

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Table 1

Methods of Determination of Enzyme Activities and Metabolites

Enzyme	Method (Ref.)
Glucose-6-phosphate dehydrogenase	Loehr and Waller, 1974
6-Phosphogluconate dehydrogenase	King, 1974
Glucose-6-phosphate	Lang and Michal, 1974
D-Gluconate-6-phosphate	Haid, 1974
Ribulose-5-phosphate	Racker, 1974

Table 2

Ribulose-5-phosphate levels in *Ephestia cautella* pupae
exposed to controlled atmospheres for 24 and for 48 h

N ₂	O ₂	CO ₂	Ribulose-5-phosphate μMole/g insect	
			24 h (±S.E.)	48 h (±S.E.)
80	20	--	0.44 (±0.065)	0.077
90	10	--	0.496 (±0.057)	
99	1	--	0.248 (±0.033)	
--	20	80	0.152 (±0.016)	
--	10	90	0.132 (±0.017)	0.089 (±0.027)
"Untreated"			0.107 (±0.031)	

Table 3

Changes of reaction velocity for the decarboxylation
of 6-phosphogluconic acid at a starting pH 7.2
with decreasing pressures of CO₂

<u>CO₂, %</u>	<u>HCO₃, M</u>	<u>(nanoMole/min/mg)</u>
2	0.007	12.7
10	0.04	4.8
20	0.07	4.5
30	0.11	4.9

Table 4

Reaction rates at various carbon dioxide pressures
 (±S.E.; 25°C; pH, 7.4)

<u>CO₂, %</u>	<u>Pyruvate → Malate</u>	<u>Malate → Pyruvate</u>
	<u>----- (nanomoles/min/mg protein) -----</u>	
0	-	6.335 (±0.450)
20	1.614 (±0.000)	2.975 (±0.136)
30	1.881 (±0.088)	-
40	2.614 (±0.260)	-
50	1.461 (±0.000)	-
60	-	1.578 (±0.072)
66	1.613 (±0.171)	1.627 (±0.000)

Table 5

Glutathione levels in pupal tissue ($\mu\text{Mole/g}$) after treatment in
atmospheres of varied compositions

<u>Gaseous composition</u>			<u>Exposure time</u>		
<u>N₂</u>	<u>O₂</u>	<u>CO₂</u>	<u>24 h</u>	<u>48 h</u>	<u>72 h</u>
80	20	0	1.65 $\pm$ 0.07		
90	10	0	1.52 $\pm$ 0.01		
99	1	0	1.36 $\pm$ 0.05		
0	20	80	1.24 $\pm$ 0.08		
0	10	90	1.17 $\pm$ 0.02	0.98 $\pm$ 0.07	
Untreated			1.49 $\pm$ 0.06		0.86 $\pm$ 0.005

Table 6

Recuperation to 24 h exposure to 90% CO₂
(in $\mu\text{Mole/g}$ glutathione)

<u>Recuperation time</u>			
<u>0 h</u>	<u>24 h</u>	<u>48 h</u>	<u>72 h</u>
1.17 $\pm$ 0.02	1.05 $\pm$ 0.001	0.83 $\pm$ 0.03	1.68 $\pm$ 0.02

Table 7

Glutathione levels after application of MeBr

<u>MeBr</u> <u>cf $\frac{\text{g h}}{\text{m}^3}$</u>	<u>Untreated</u>	<u>Prior treatment</u> <u>with 90% CO₂</u> <u>(24 h)</u>
10	1.02 $\pm$ 0.03	1.08 $\pm$ 0.04
20	0.56 $\pm$ 0.1	0.75 $\pm$ 0.04

Figure 1. Mortality of different stages of *Ephestia* larvae when exposed to 20% O₂:80% CO₂ for different times.

Figure 2. Mortality of more precisely aged larvae of *Plodia* and *Ephestia* when exposed 16 hours to 20% O₂:80% CO₂

$\mu\text{l O}_2$ consumed/mg/hr

Figure 3. O_2 consumption of *Plodia* larvae when reared on diets with and without JH. Stippled bar represents additional O_2 consumed when CO_2 allowed to accumulate up to 1% in the Warburg flask.

Figure 4. Effect of starvation on the growth and longevity of 6-8 mg *Plodia* larvae.

Table 8 Effects of Starvation on Respiration of 6-8 mg Plodia Larvae

Experimental <u>Conditions</u>	QO_2 <u>ul/hr/mg tissue</u>
0 hrs.	5.0
16 hrs Starved	2.4
24 hrs Starved	2.6
48 hrs Starved	2.0
24 hrs Starved, Refed 24 hrs	2.7

Table 9 Interaction of Starvation with CO₂ Treatments of 6-8 mg Plodia Larvae.

Condition	Hours					Percentage after 192 hours		Relative Growth*	
	0	13	26	39	52	192	Mortality		Pupation
1	:	:	:	Fed	:	:	0	100	+++
2	:	:	:	Starved	:	:	83%	17%	-
3	:	CO ₂	:	Fed	:	:	100%	-	-
4	:	Starved	CO ₂	:	:	:	83%	17%	+
5	:	Starved	:	CO ₂	:	Fed	78%	22%	-
6	:	Starved	:	:	CO ₂	Fed	33%	67%	+
7	:	Starved	:	:	Fed	:	0%	100%	+++
8	:	Starved	:	:	Fed	:	4%	96%	++
9	:	:	Starved	:	:	Fed	4%	96%	+
10	:	Starved	:	Fed	:	:	0%	100%	+++
11	:	Starved	:	:	Fed	:	0%	100%	+++
12	:	:	Starved	:	Fed	:	0%	100%	++

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*Relative growth occurring during the period between 0 and 192 hours.

Table 10 Changes in Oxidative Metabolism During Growth and Development of Last Instar Plodia Larvae.

Developmental		Pyr-mal oxidation		Suc oxidation		Live respiration	
Stage	Mitochondrial N	uatoms O ₂ /hr/mg M _w N	uatoms O ₂ /hr/mg M _w N	uatoms O ₂ /hr/mg M _w N	uO ₂ /hr/mg live weight		
<u>4th instar</u>							
2.5 - 3.0 mg	1.26 ± 0.07	224.7 ± 13.7	69.9 ± 3.4	5.2 ± 1.1			
<u>5th instar</u>							
3.0 - 5.0 mg	1.24 ± 0.09	230.4 ± 7.2	86.8 ± 6.0	6.4 ± 1.1			
6.0 - 8.0 mg	1.33 ± 0.07	222.0 ± 10.9	98.1 ± 1.1	5.2 ± 0.6			
9.0 - 12.0 mg	0.96 ± 0.08	184.8 ± 8.7	109.0 ± 7.7	3.8 ± 0.6			
13.0 - 16.0 mg	0.80 ± 0.05	163.4 ± 5.3	122.8 ± 4.4	2.9 ± 0.5			
Wandering	0.75 ± 0.07	72.3 ± 6.3	59.5 ± 4.7	---			