cholesterol in MHVA-treated rats was 84.8 ± 4.1 mg/100 ml vs 88.6 ± 5.8 mg/100 ml in the control group ($p > 0.10$, t-test). The liver cholesterol content in MHVA-treated rats was also similar to that of control rats (265 ± 17 mg/100 g vs 260 ± 4 mg/100 g of fresh liver in the control group). On the contrary, in rats with a 1% cholesterol diet, the hepatic cholesterol content showed an 8% increase. Table I shows that a diet 1% cholesterol plus 0.5% sodium deoxycholate, offered for 48 h, produced 60% inhibition of acetate incorporation into hepatic cholesterol. The administration of MHVA to cholesterol-fed rats produced 77% inhibition. MHVA-treated rats showed 69% inhibition of acetate incorporation into cholesterol. Diosgenin-fed rats showed, as indicate previous reports[9], increased incorporation of acetate into cholesterol. The administration of MHVA to diosgenin-fed rats partly reversed the action of diosgenin. Finally, as MHVA inhibits both acetate and mevalonate incorporation into cholesterol, this effect does not correlate with a direct action on HMG CoA reductase activity. This was confirmed when the enzyme was assayed in liver microsomes. MHVA did not inhibit the activity of HMG CoA reductase (Table II). On the other hand, a 1% diosgenin diet produced a moderate, statistically significant increase in the activity of the enzyme. Also, in agreement with previous reports[13], a high cholesterol diet inhibited the activity of HMG CoA reductase.

Table II. Activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of MHVA-treated, diosgenin-fed or cholesterol-fed rats

Group	Activity of HMG CoA reductase	
Control (9)[a]	5.47 ± 0.52[b]	
MHVA (4)	5.31 ± 0.28	$p > 0.10$
1% diosgenin (10)	9.48 ± 3.68	$p < 0.005$
1% cholesterol (4)	2.15 ± 0.15	$p < 0.005$

The animals indicated received for 4 days before their sacrifice 1.88 m*M* per 100 g of body weight of MHVA, dissolved in phosphate buffer pH 7.8, or the equivalent volume of phosphate buffer alone. The animals of groups 3 and 4 received the diets indicated 48 h before their sacrifice. Reductase activity is expressed as nm of mevalonate formed per h per mg of microsomal protein. Generally 4 trials were run from each animal. The assay system consisted of 0.8 ml containing 100 m*M* phosphate buffer pH 7.2, 3 m*M* $MgCl_2$, 3 m*M* NADP, 10 m*M* glucose 6 phosphate, 2.5 units glucose 6 phosphate dehydrogenase, 50 m*M* reduced glutathione, 0.2 m*M* (3-^{14}C)-HMG CoA and 0.7–0.9 mg microsomal protein. [a] Number of rats of each group is indicated in parenthesis. [b] Standard error of the mean.

Discussion. Although we cannot ascertain the mechanism of action of MHVA with the data presented in this paper, the results show that this valeric acid derivative inhibits hepatic cholesterol synthesis from acetate or mevalonate without inhibiting HMG CoA reductase activity. On the other hand, diosgenin-fed rats showed a moderate increase in HMG CoA reductase activity compared with the 3-fold increase in acetate incorporation into cholesterol, with no change in mevalonate incorporation into cholesterol[9]. Therefore diosgenin increases cholesterol biosynthesis in the liver at least as a result of increasing the activity of the rate-limiting step in biosynthetic pathway. On the contrary, MHVA inhibits hepatic cholesterol synthesis, but this effect is not a direct action on the activity of HMG CoA reductase. We cannot assure the MHVA action mechanism at the present state of our research, but the effect reported here contributes to the developing of new orally-active agents for human therapy in cholesterol metabolism disorders.

[13] D. J. SHAPIRO and Y. W. RODWELL, J. biol. Chem. *246*, 3210 (1971).

Effect of Benzimidazole on Nicotinamide Adenine Dinucleotide Phosphate Phosphomonoesterase Activity in Wheat Leaves[1]

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Summary. Nicotinamide adenine dinucleotide phosphate phosphomonoesterase was isolated and partially purified from wheat (*Triticum aestivum* L. var. Selkirk) leaves. The enzyme had K_{NADP} value of 1.4×10^{-4} *M* and a pH optimum of 5.9. *In vitro* activity of this enzyme was unaffected by precursors of NAD (nicotinamide and nicotinic acid) or cytokinins (kinetin and benzimidazole). However, when detached wheat leaves were treated with solutions of these compounds, the precursors lowered the specific activity while the cytokinins enhanced the activity. It is suggested that spatial separation and compartmentation of the enzyme and its substrate NADP account for the similar effect of benzimidazole on both.

Earlier investigations in this laboratory into the effects of benzimidazole on the metabolism of excised leaves presented a varied and complex pattern of its influences[3]. Amongst these are the effect of benzimidazole on structure and integrity of chloroplasts[4] and the metabolism of nicotinamide nucleotides[5]. A marked increase was reported in NADP content and in the NADP/NAD ratio in detached leaves of wheat (*Triticum aestivum* var. Selkirk) treated with solutions of benzimidazole or kinetin[6]. Benzimidazole treated leaves fed with radioactive precursors accumulated radioactivity in NADP > NAD, while leaves floated on water accumulated the radioactivity in NAD > NADP[5,7]. However NAD but not NADP was reported to accelarate the senescence of chloroplasts in plasmolyzed protoplasts of *Elodea* leaves[8]. YOSHIDA's work was extended in this laboratory to detached unplasmolyzed leaves of *Elodea* and wheat. In both cases NAD but not NADP accelerated the senescence (as measured by chlorosis) of detached leaves and benzimidazole overcame this effect[7]. These studies suggest that the senescence of detached wheat leaves and the effect of cytokinins on senescence are directly or indirectly connected to the ratio of the concentrations of NAD and NADP in wheat leaves. NAD kinase (EC 2.7.1.23) which phosphorylates NAD to NADP[9] and NADP-phosphatase (EC 3.1.3.2) which hydrolyzes NADP to NAD[10,11] appear to play important

roles in regulating the ratio of NAD to NADP. In this investigation the effect of kinetin and benzimidazole on the activity of NADP-phosphatase and on the levels of the enzyme in detached wheat leaves treated with benzimidazole and kinetin were studied.

Selkirk wheat (*Triticum aestivum* L. var. Selkirk) was grown in the greenhouse for 8 to 11 days and the primary leaves were used.

Enzyme isolation was carried out in a cold room maintained under 5 °C. Leaves were washed and homogenized with 1.5 volumes (w/v) of 50 m*M* *Tris* acetate buffer, pH 7.3, containing 1.0 m*M* EDTA for 5 min at 0 to 5 °C. The homogenate was squeezed through 4 layers of cheesecloth and centrifuged (as all subsequent centrifugations were) at $20{,}000 \times g$ for 20 min. The supernatant was subjected to a step-wise fractionation by the addition of solid ammonium sulphate and the suspension was stirred slowly for 2 h after each step. The protein precipitated between 25% to 80% ammonium sulphate saturation was collected by centrifugation and redissolved in a volume of 50 m*M* *Tris* acetate buffer, pH 7.3 containing 1.0 m*M* EDTA equal to $^1/_2$ the volume of the initial crude extract. An equal volume of 2% protamine sulphate was added slowly with constant stirring to the first ammonium sulphate fraction. The denatured protein was removed by centrifugation. The supernatant was dialyzed overnight against 2 changes of a total of 20 volumes of 50 m*M* *Tris* acetate buffer at pH 6.0 containing 1.0 m*M* EDTA. The precipitate formed during dialysis was removed by centrifugation and discarded. The dialysate was fractionated by the addition of solid ammonium sulphate and the protein precipitating between 45% to 65% ammonium sulphate saturation was collected by centrifugation. It was dissolved in a small volume of 50 m*M* *Tris* acetate buffer, pH 7.3, containing 1.0 m*M* EDTA and dialyzed for 48 h against 2 changes of the same buffer. The enzyme was centrifuged after dialysis to remove any insoluble proteins present[10,11].

The data on the intracellular localization of NADP phosphomonesterase are shown in Table I. NADP phosphatase activity could not be detected in the highly purified chloroplasts isolated by the 'laceration technique' of MACHE and WAYGOOD[12]. On the other hand, the enzyme activity was apparently restricted completely to the supernatant.

Nicotinic acid and nicotinamide, precursors of NAD biosynthesis[5,7] had little or no effect on the in vitro activity of NADP-phosphatase. Similarly benzimidazole and kinetin which influence the levels of NADP in the wheat leaves[6] were without effect. The results are shown in Table II.

Excised leaves in 5 g batches were washed, dried with paper towels and floated for 3 days in glass trays which contained either 250 ml of distilled water or a solution of one of the following chemicals: nicotinic acid (1.0 m*M*); nicotinamide (1.0 m*M*); NAD (1.0 m*M*); benzimidazole (424 μM) or kinetin (93 μM). The trays were covered with Saran Wrap and placed in a growth chamber at 21 °C under continuous illumination at a light intensity of 1500 ft c.

The treated leaves were rinsed several times with distilled water and the enzymes extracted as described earlier. The result of the effect of various treatments on the level of NADP phosphomonosterase in detached wheat leaves are shown in Table III. The levels of NADP phosphatase activity decreased in the leaves floated on

Table I. Intracellular localization of NADP Phosphomonoesterase[a].

Fraction	Total enzyme units[b]	
	Experiment I	Experiment II
Chloroplast sonicate	0	0
Supernatant	7.5	8

[a]The assay system contained *Tris* acetate buffer pH 6.5, 100 µmoles; EDTA 0 1 µmole; ethanol, 180 µmoles; alcohol dehydrogenase from Baker's yeast in excess; enzyme preparation 0.1 ml; and NADP, 0.5 µmole in a total volume of 3.0 ml. The reaction was started with the addition of NADP. The NAD formed was reduced by the alcohol dehydrogenase system[15] to NADH and the increase in absorbance at 340 nm was measured using silica cuvettes of 1 cm light path in a Unicam SP800 automatic recording spectrophotometer. [b]One enzyme unit is defined as the amount of enzyme which catalyses the formation of 1 µmole of product in 5 minutes.

Table II. Effect of nicotinic acid, nicotinamide, benzimidazole and kinetin on NADP phosphomonoesterase

Compounds	Concentration (*M*)	Activity
Nicotinic acid	10^{-4}	96.6
	2×10^{-4}	93.3
	2×10^{-3}	93.3
	4×10^{-3}	90
Nicotinamide	10^{-4}	95
	10^{-3}	93.3
	2×10^{-3}	93.3
Kinetin	3.1×10^{-6}	100
	7.7×10^{-6}	100
	1.5×10^{-5}	103
	3.1×10^{-5}	106.6
Benzimidazole	1.4×10^{-5}	100
	7.0×10^{-5}	93.3
	1.4×10^{-4}	93.3

Assay system contained *Tris* acetate buffer, pH 6.0, 100 µmoles; EDTA 0.1 µmole; and 0.1 ml enzyme preparation in a total volume of 3.0 ml. No preincubation with added compounds. The reaction was started by the addition of enzyme preparation and incubated at 37 °C. It was terminated by heating at 100 °C for 2 min. Orthophosphate liberated was determined at 650 nm as a phosphomolybdate complex reduced by ascorbic acid according to the method of WAYGOOD[16]. The system contained 1.0 *M* Am. acetate (pH 4.0) 2.0 ml, 5% Am. molybdate 0.5 ml, an aliquot of *Pi*, 1% ascorbic acid 1.0 ml, made up to a final volume 10 ml with distilled H_2O.

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[3] M. KAPOOR and E. R. WAYGOOD, Can. J. Biochem. *43*, 153 (1965).

[4] E. R. WAYGOOD, Plant Physiol. *40*, 1242 (1965).

[5] H. R. GODAVARI and E. R. WAYGOOD, Can. J. Bot. *48*, 2267 (1970).

[6] D. MISHRA and E. R. WAYGOOD, Can. J. Biochem. *46*, 167 (1968).

[7] E. R. WAYGOOD, D. MISHRA and H. R. GODAVARI; in *Biochemistry and Physiology of Plant Growth Substances* (Eds. F. WIGHTMAN and G. SETTERFIELD, The Runge Press, Ottawa 1968), p. 1495.

[8] Y. YOSHIDA, Protoplasma *54*, 476 (1961).

[9] A. KORNBERG, J. biol. Chem. *186*, 779 (1950).

[10] A. KORNBERG and W. E. PRICER, J. biol. Chem. *186*, 557 (1950).

[11] G. FORTI, C. TOGNOLI and B. PARISI, Biochim. biophys. Acta *62*, 251 (1962).

[12] R. MACHE and E. R. WAYGOOD, FEBS Lett. *3*, 89 (1969).

Table III. NADP phosphatase[a] activity in detached wheat leaves treated with nicotinic acid, nicotinamide, NAD, benzimidazole or kinetin

	Protein (mg/ml)		Specific activity[b] (EU/mg protein)	
	Experiment No. I	II	Experiment No. I	II
Immediately detached	11.0	10.2	2.19	2.3
Water	9.8	9.5	2.33	2.40
Nicotinic acid	9.5	9.2	1.77	2.22
Nicotinamide	9.6	9.0	3.27	2.89
NAD	10.0	9.1	1.44	1.89
Benzimidazole	10.2	9.8	2.51	3.09
Kinetin	10.1	9.8	3.53	3.38

[a]Spectrophotometric assay for NAD formed in 33 m*M Tris* at pH 6.0.
[b]One enzyme unit is defined as the amount of enzyme which catalyses the formation of 1 µmole of NAD in 5 min.

NAD and nicotinic acid. The leaves floated on kinetin and benzimidazole showed an increase in NADP phosphatase activity.

The NADP-phosphatase in this study resembles the enzyme from pea leaves isolated by FORTI *et al*[11]. Both are acid phosphatases which hydrolyze NADP at high rates. The K_{NADP} value of the acid phosphatase from wheat leaves was 1.4×10^{-4} *M* in this study (c.f. 3×10^{-4} *M* for the pea leaf enzyme). Orthophospate was found to be a competitive inhibitor of both wheat leaf phosphatase and pea leaf phosphatase. The K_{iq} ($K_{i\ (Pi)}$) was 1.7×10^{-4} *M* in this study.

The specific activity of NADP-phosphatase in excised wheat leaves treated with solutions of kinetin or benzimidazole increased approximately 20% while the leaves treated with solutions of nicotinic acid or NAD lost about 20% of the specific activity. However, MISHRA and WAYGOOD[6] reported that wheat leaves treated with benzimidazole or kinetin had an increas ed NADP content and a fairly constant NAD-NADP ratio in fresh leaves. As the NADP phosphatase was an active enzyme even in the crude wheat leaf extract this indicates that a regulatory factor other than the enzyme level controls the NAD/NADP ratio in the leaves. If so, an increase in the specific activity of NADP-phosphatase in vitro will not necessarily cause the decrease of NADP content in vivo. This regulation is facilitated further by the compartmentation of the enzyme and substrate in separate loci. The main pool of NADP in leaves treated with the cytokinins is possibly in the chloroplasts while NADP-phosphatase was located outside the chloroplasts. Benzimidazole by maintaining the structural integrity of the chloroplasts prevents 'leaching' of the NADP from chloroplasts and hence the enzymic hydrolysis to NAD. Benzimidazole also favours the synthesis of NADP (presumably from NAD). Benzimidazole sustitution for the nicotinamide moiety of NAD has been reported by KAPOOR and WAYGOOD[13]. This substitution reaction together with enhanced transhydrogenase[14] and presumably also NAD kinase activity and the spatial separation of NADP-phosphatase from its substrate possibly explain the seemingly contradictory situation of an increase in the levels of both NADP and NADP-phosphatase in leaves treated with benzimidazole[15].

[13] M. KAPOOR and E. R. WAYGOOD, Can. J. Biochem. *43*, 165 (1965).
[14] C. E. JOU, M. Sci. Thesis, University of Manitoba (1970).
[15] E. RACKER in *Methods of Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN; Academic Press, New York, 1955), vol. 1, p. 500.
[16] E. R. WAYGOOD, Can. J. Res. *26*c, 461 (1948).

Location of an Eye Mutant in the Onion Fly *Hylemya antiqua* Meigen Using a Pericentric Chromosome Inversion

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Summary. With the use of a pericentric inversion in chromosome 3, an eye color mutant in the onion fly was located in chromosome 3. No recombination occurs in males; 40.2% recombination was observed in females. This linkage through the male facilitates further cytogenetic research on structural aberrations involving chromosome 3.

Genetic research on the onion fly *Hylemya antiqua* Meigen has been undertaken in the context of genetic insect control. Several X-ray induced chromosomal rearrangements (translocations and inversions) associated with reduced fertility have been studied[1–3]. Unfortunately, no morphological markers have yet been isolated. Such markers are useful for the isolation of homozygous translocations or inversions; cytogenetic analysis alone being more time-consuming. Recently we have received a white eye (*ww*) marker stock from Canada[4]; the wild type flies are red eyed (w^+w^+). This mutant is not sex-linked[5], thus it will be located on 1 of the 5 autosomes ($2n=12$). With the aid of a pericentric inversion, it was possible to assess on which chromosome this white eye marker is located.

Inversion *In(3)2* was used in which chromosome 3 is involved[1], see Figure. In mitotic metaphases, the inversion heterozygous (*In*/+) karyotypes could be discriminated from the +/+ types on the basis of the different arm-ratio of the inverted chromosome in combination with the transposition of a secondary constriction from the short arm of the normal chromosome to the long arm of the inverted chromosome (Figures c and e). In meiotic stages, the inversion heterozygous types could be identified by the presence of a clear ring or bump at one end of the chromosome 3 bivalent (Figures d and f). The fertility is approximately 30% reduced (70% egg hatch), as measured from test-crossed inversion heterozygous (*In*/+) females (chiasmate), while *In*/+ males have a normal fertility due to the absence of chiasmata.