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EFFECT OF 2, 4-D ON NAD+-INCORPORATING ACTIVITY OF GREEN GRAIN SEEDLINGS[1,2]

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Abstract

NAD+-incorporating activity of stored crude chromatin and 1.7 M sucrose centrifugation-purified chromatin of various portions of etiolated mungbean seedlings with or without 2, 4-D treatment was determined and expressed in three different ways. The order of specific activity expressed as nmol NAD+ incorporated/mg protein was hypercotyl > leaf > hypocotyl > root; as nmol NAD+ incorporated/100g fresh tissue, the order was leaf > hypercotyl > hypocotyl, root; as nmol NAD+ incorporated/mg DNA, the order was hypercotyl ≫ hypocotyl, leaf > root. 2, 4-D (2.5 mM, pH 6.6) treatment decreased the enzyme activity of hypercotyl crude chromatin, while increased the enzyme activity of hypocotyl crude chromatin.

For normal tissues, variance analysis of NAD+-incorporation activity showed that: (1) there is no significant difference between upper parts (from 0.5 cm below cotyledons upward including cotyledons and leaves) and lower parts (from 1.0 cm below cotyledons to roots) of seedlings; (2) there is a very significant difference among various batches of mungbean seedlings of the same variety; (3) there is a very significant interaction between parts and batches. For 2, 4-D treated samples, variance analysis of NAD+-incorporation activity showed that: (1) there is a very significant difference between upper and lower parts; (2) there is a very significant difference of effects of concentrations of 2, 4-D; (3) there is no significant interaction between parts and concentrations of 2, 4-D. When incorporation rate of NAD+ (rate of NAD+ treated samples minus controls) of whole mungbean seedlings was plotted against natural logarithm of 2, 4-D concentrations (ppm), a curve, $y = -1067.6 + 641.1x - 60.6x^2$, could be fitted at a confidence level of 95%.

Key words: NAD+-incorporation; mungbean; 2, 4-D; chromatin.

Introduction

NAD+-incorporating activity in tissues of higher plants has been described (Lin, 1975, 1976; Lin and Chang, 1976, 1980; Lin and Kuo, 1981; Payne and Bal,

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1976). In these reports the occurrence and properties of the incorporation activity, properties of NAD^+-incorporation product(s), some kinetic properties of NAD^+-incorporating activity were presented. However the biological function of this enzyme activity is still not known exactly. Since the determination of initial velocity of NAD^+-incorporation has succeeded (Lin and Kuo, 1981), the basis of studying the biological significance of NAD^+-incorporation is sound. 2,4-Dichlorophenoxyacetic acid (2,4-D) is an auxin type synthetic herbicide and its effect on the physiology and biochemistry of etiolated soybean seedlings is well documented (Chen *et al.*, 1975; Guilfoyle *et al.*, 1975; Holm and Abeles, 1968; Key *et al.*, 1960; Key *et al.*, 1966; Murray and Key, 1978).

Hypocotyl of etiolated soybean seedlings, usually is matured, but 2,4-D treatment induces enlargement of mitochondria, changes of chemical composition and metabolism such as: increase of nucleotides and phospholipids; increasing of phosphorylation reaction (Key *et al.*, 1960). Key *et al.* (1966) also pointed out that after 2,4-D treatment, biosynthesis of nucleic acids and protein of young tissue (hook) is inhibited; while that of the matured tissue of hypocotyl is enhanced obviously and there is large amounts of ribonucleic acids, especially ribosomal RNA accumulated. Chen *et al.* (1975) found that after 2,4-D treatment nucleoli enlarge; nuclear protein and RNA increase; and the activity of DNA-dependent RNA polymerase I which is responsible for biosynthesis of ribosomal RNA is 2.5 times that of control. Guilfoyle *et al.* (1975) presented evidences which showed that enhancement of biosynthesis of ribosomal RNA induced by 2,4-D treatment is due to the direct activation of DNA-dependent RNA polymerase I instead of activation of ribosomal RNA genes.

In this report the effect of 2,4-D on the levels of NAD^+-incorporating activity of mungbean seedlings is presented as an indication that this enzyme activity may involve in physiological and biochemical functions of the young plant.

Materials and Methods

Materials

Seeds of mung bean (*Phaseolus radiatus*) were purchased at local market of Nankang, Taipei. The following compounds were obtained from commercial sources: ^{14}C-(adenine-U)-NAD^+ (271 mCi/mmole) from The Radiochemical Centre, Amersham, Buckinghamshire, U. K.; orcinol, Tris(hydroxymethyl)aminomethane, oxidized form of nicotinamide adenine dinucleotide (β-NAD^+), bovine serum albumin and yeast *t*-RNA (type III) from Sigma Chemical Co.. St. Louis, Missouri, USA; Folin Ciocalteau reagent, acetaldehyde, $FeCl_3$, $HClO_4$, HCl and acetic acid from Wako Pure Chemical Ltd., Osaka, Japan; exherring sperm DNA from Calbiochem, Los Angeles, California, USA; trichloroacetic acid, diphenylamine, 2,5-piphenyloxazole (PPO) and

1,4-bis(2-(5-phenyloxazolyl))-benzene (POPOP) from E. Merck, Darmstad, Germany.

Germination Procedure

Seeds of ***Phaseolus radiatus*** were soaked in 0.1-0.5% H_2O_2 for 20 min and then in distilled water overnight. Swollen seeds were put on petri dishes with wet cheese cloth and then transferred to a growth incubator set at 25 ± 0.5°C. Germination proceeded in the dark for 7 days for control group. For treatment group, seedlings of 6-day old were sprayed with 2,4-D (pH 6.6) of different concentrations and were harvested after 24 hours. The cheese cloth was kept wet during germination period.

Preparation of Crude Chromatin

This was done mainly according to Huang and Bonner (1965). About 10–100 gm of fresh 7-day old etiolated seedlings of *P. radiatus* was homogenized at 4°C with a fruit blender in 5 times (v/w) of grinding medium consisting of 0.25 M sucrose, 0.05 M Tris-HCl buffer pH 8.0 and 1 mM $MgCl_2$. The homogenate was then filtered successively first through a single layer, then two layers and finally four layers of cheese cloth. The homogenate was centrifuged at 4,300 g for 30 min. The pellets of crude chromatin were scraped from the underlying layers. Crude chromatin was then homogenzied in distilled water with a Potter-Elvehjem hand homogenizer. For immediate use, the homogenized chromatin was dialyzed (crude chromatin) and used as an enzyme source after storage for 2 days. For further purification, the dialyzed crude chromatin was subjected to the following treatment.

Partial Purification

The dialyzed crude chromatin was layered on top of about 40 ml of the grinding medium stated above, except that 0.25 M sucrose was replaced by 1.78 M sucrose. Centrifugation was carried out at 12,000 g for 30 min. The layer floating on top after centrifugation was discarded while both middle and bottom layers were collected, dialyzed (partial purified chromatin), and stored for 2 days before being used.

Storage of the Crude Chromatin or Partial Purified Chromatin

Either crude chromatin or partial purified chromatin was stored in a reaction mixture without substrate at 7°C for 2 days before enzyme activity was assayed. Sixty μl of chromatin containing 400 μg or less protein was mixed with 20 μl of 0.5 M Tris-HCl, pH 7.4, 10 μl of 0.25 M $MgCl_2$ and 135 μl of double distilled water and kept at 7°C for 2 days. Then 5–10 μl of ^{14}C-(adenine-U)-NAD^+ having 20,000–60,000 cpm and 20–15 μl of 2.5 mM unlabelled β-NAD^+ were added simultaneously

to the stored chromatin solution to make a final volume of 250 μl to start the enzyme reactions.

Determinations of Protein, DNA and RNA

The protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Diphenylamine assay (Burton, 1956) was used for DNA determinations with exherring sperm DNA as standard.

Orcinol assay of ribose (Dische, 1955) was used for RNA determinations with yeast *t*-RNA (type III) as standard.

Assay of Incorporation

The reaction mixture contained 40 mM Tris-HCl. ph 7.4, 10 mM $MgCl_2$, 5–10 μl of ^{14}C-(adenine-U)-NAD^+ with 20,000–60,000 cmp together with 20–15 μl of 2.5 mM unlabelled NAD^+, and stored crude chromatin or stored partial purified chromatin with 400 μg or less protein in a final volume of 250 μl. The reaction was carried out at 25°C for 1–5 min or for 15 min. Chromatin preparation preheated at 80°C for 10 min was used for control tests. At the end of the reaction. the mixtures were combined with equal volume of 20% (w/v) trichloroacetic acid to stop enzyme reactions. Fifty μl of each reaction mixture was pipetted out onto a Whatman No. 1 filter paper disk, and the disks were batch washed in cold 5% trichloroacetic acid with 10% $Na_4P_2O_7$, cold 5% trichloroacetic acid, 95% ethanol, and ether, mainly described by Yoneda and Bollum (1965). Radioactivity on the dried disks was determined by counting in 0.4% PPO (w/v) and 0.024% POPOP (w/v) in toluene in a Beckman liquid scintillation counter LS-100C. All data after correction for control tests were calibrated using a standard curve with counting efficiency versus amounts of protein in reaction mixtures.

Results

NAD^+-incorporating Activity of Various Tissues of Mungbean Seedlings

Table 1 shows specific NAD^+-incorporating activity with three different expression units of various tissues of mungbean seedlings. This work was done during October, 1979. Weight ratios of protein: DNA for crude chromatin of hypercotyl, leaf, hypocotyl and root were 60.0, 14.9, 41.4, and 26.0, respectively. The protein contents of crude chromatin (μg/g fresh tissue) were 374, 920, 767, and 1280; and DNA contents of crude chromatin (μg/g fresh tissue) were 6.24, 61.7, 18.5, and 49.0.

The order of specific activity expressed as nmol NAD^+-incorporated/mg protein was hypercotyl > leaf > hypocotyl > root; as nmol NAD^+-incorporated/100g fresh tissue. the order was leaf > hypercotyl > hypocotyl, root; as nmol NAD^+-incorporated/mg DNA, the order was hypercotyl $\gg$ hypocotyl, leaf > root.

Table 1. *NAD^+-incorporating activity of stored crude chromatin of various tissues of mungbean seedlings*

Crude chromatin containing 400 μg protein and NAD^+ with a final concentration of 0.3 mM was used. Other components were the same as those described in "Materials and Methods". The reaction time was 4 min. Each value is the average of duplicate determinations.

Expression of enzyme activity	Leaf	Hypercotyl	Hypocotyl	Root
nmol NAD^+ incorporated per mg protein	0.180	0.495	0.128	0.068
nmol NAD^+ incorporated per 100g fresh tissue	29.2	18.5	9.83	8.66
nmol NAD^+ incorporated per mg DNA	4.8	29.7	5.32	1.76

Table 2 shows results of a similar experiment in which effect of 2, 4-D treatment on NAD^+-incorporating activity was examined. This work was done in August 1980 using different batch of mungbean seeds. All the specific enzyme activities of various tissues of mungbean seedlings were much higher than those presented in Table 1. However, the relative order of specific activity of three different tissues was quite similar to those in Table 1, no matter what kind of expression is used. For nmol NAD^+-incorporated/mg protein, hypercotyl>leaf>hypocotyl; for nmol NAD^+-incorporated/100 g fresh tissue, leaf > hypercotyl > hypocotyl; for nmol NAD^+-incorporated/mg DNA, hypercotyl > leaf > hypocotyl. The effect of 2, 4-D on the enzyme activities of both hypercotyl and hypocotyl was obvious and distinct. 2, 4-D treatment decreased the enzyme activity of hypercotyl crude chromatin no matter what kind of expression was used, while 2, 4-D treatment increased the enzyme activity of hypocotyl chromatin to a level almost close to that of hypercotyl under the same treatment.

Table 2. *Effect of 2,4-D treatment on NAD^+-incorporating activity of stored crude chromatin of various tissues of mungbean seedlings*

Crude chromatin containing 400 μg protein and NAD^+ with a final concentration of 0.5 mM was used. Reaction time was 40 seconds. 2, 4-D concentration was 2.5 mM (or 552 ppm).

Expression of enzyme activity	Leaf		Hypercotyl		Hypocotyl	
	Control	2, 4-D treated	Control	2, 4-D treated	Control	2, 4-D treated
nmol NAD^+ incorporated per mg protein	2.60	N. D. [1]	3.87	2.30	0.28	1.9
nmol NAD^+ incorporated per 100g fresh tissues	480	N. D.	384	227	15.3	104
nmol NAD^+ incorporated per mg DNA	13	N. D.	23	12	1.20	9.8

[1] N. D. : Not determined

Effect of 2,4-D treatment on NAD+-incorporating activity of stored partial purified chromatin of whole mungbean seedlings

Once the effect of 2,4-D treatment on NAD+-incorporating activity was oberved, further experiments were done. Table 3 and Table 4 show results of effect of 2,4-D on NAD+-incorporating activity of stored partial purified chromatin of whole mungbean seedlings. These experiments were carried out in December 1980. Although variations in data of comparable sets of experiments existed, the effect of 2,4-D on average enzyme activity of both middle layer and bottom layer preparations was obvious and distinct. 2,4-D treatment decreased the enzyme activity of stored middle layer of partial purified chromatin but increased that of bottom layer no matter the specific enzyme activity was expressed on the basis of mg protein or mg DNA. The effect of 2,4-D was consistent for short-time reactions (5 min) and longer-time reactions (15 min).

Table 3. *Effect of 2,4-D treatment on NAD+-incorporating activity of stored partial purified chromatin of whole mungbean seedlings*

Detail of control and 2,4-D treatment (2.5 mM) of germinating seedling preparations of partial purified chromatin, and incorporation were same as those described in "Methods". Each value was the average of duplicate determinations. Reaction time was 5 min.

Preparation	Treatment	nmol NAD+ incorporated per mg protein			nmol NAD+ incorporated per mg DNA		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Middle layer	Control	5.980	1.480	ND[1]	17.500	7.130	ND[1]
	2,4-D	1.790	0.521	0.275	4.550	2.440	5.750
Bottom layer	Control	1.050	1.320	0.321	2.420	6.050	5.150
	2,4-D	3.380	0.161	0.738	7.950	0.831	12.300

[1] ND: Not determined

Table 4. *Effect of 2,4-D treatment on NAD+-incorporating activity of stored partial purified chromatin of whole mungbean seedlings*

All experimental conditions were the same as in Table 3 except that reaction time was 15 min.

Preparation	Treatment	nmol NAD+ incorporated per mg protein			nmol NAD+ incorporated per mg DNA		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Middle layer	Control	5.920	4.450	ND	17.40	21.50	ND
	2,4-D (2.5mM)	1.570	1.350	0.533	4.00	6.32	11.20
Bottom layer	Control	0.976	4.800	0.683	2.26	19.99	4.17
	2,4-D (2.5mM)	6.850	0.797	1.190	16.10	4.41	19.70

Variance Analysis of NAD+-incrporating Activity of Chromatin Isolated from Upper and Lower Parts of Different Batches of Mungbean Seedlings with and without 2,4-D Treatment

Table 5 shows variance analysis of NAD+-incorporating activity of chromatin isolated from upper and lower parts of different batches of mungbean seedlings without 2,4-D treatment. It is clear that: (1) there is no significant difference between upper and lower parts; (2) there is a very significant difference among various batches of mungbean seedlings of the same variety; (3) there is a very significant interaction between parts and batches.

Table 5. *Variance analysis of NAD+-incorporating activity of chromatin isolated from upper and lower parts of different batches of mungbean seedlings*

All experimental conditions were the same as described in "Methods". Reaction time was 10 min. Upper parts stand for those parts of seedlings from 0.5 cm below cotyledons upward including cotyledons and leaves; lower parts stand for those parts of seedlings from 1.0 cm below cotyledons to roots. All seedlings were collected at the seventh day of germination. Different batches of seedlings mean those collected from seeds of the same variety germinated at different times.

Source of variance	Degree of freedom	Sum of square	Mean square	F value
Parts (P)	1	111,929.96	111,929.96	3.63NS
Batches (B)	5	2,978,001.71	595,600.34	19.30**
P×B	5	1,957,630.79	391,526.16	12.69**
Error	12	370,383.50	30,865.29	
Total	23	5,417,945.96		

NS not significant
** significant at $P < 0.01$

Table 6 shows variance analysis of NAD+-incorporating activity of chromatin isolated from upper and lower parts of different batches of mungbean seedlings sprayed with 2,4-D of various concentrations. Three conclusions can be reached: (1) there is a very significant difference between upper and lower parts; (3) there is a very significant difference of concentrations of 2,4-D; (3) there is no significant interaction between parts and concentrations of 2,4-D.

Effect of Treatment with Various Concentrations of 2,4-D

Figure 1 and Fig. 2 briefly summarize the effect of various concentrations (ppm) of 2,4-D on NAD+-incorporating activity of chromatin preparations of mungbean seedlings. In Fig. 1, relative incorporation rate of NAD+ (% of control) was plotted

Table 6. *Variance analysis of NAD+-incorporating activity of chromatin isolated from upper and lower parts of different batches of mungbean seedlings*

All experimental conditions were the same as in Table 5 except that 2,4-D of various concentrations was sprayed on 6-day-old seedlings 24-h before collection.

Source of variance	Degree of freedom	Sum of square	Mean square	F value
Parts (P)	1	53,817.06	53,817.06	11.68**
Conc. (C)	5	561,933.47	112,386.69	24.39**
P×C	5	53,449.10	10,689.82	2.32^NS
Error	12	55,286.57	4,607.21	
Total	23			

NS not significant
** significant at $P < 0.01$

against natural logarithm of 2,4-D concentrations (ppm); while in Fig. 2, incorporation rate of NAD+ (rate of 2,4-D treated samples minus controls) was plotted against natural logarithm of 2,4-D concentrations (ppm). In Fig. 1, curves of upper parts, lower parts, and whole seedlings are presented; each had a maximum peak. High concentrations of 2,4-D seemed to inhibit relative NAD+-incorporation activity of upper and lower parts and whole seedlings completely.

In Fig. 2 data of whole mungbean seedlings can fit equation $y = -1067.6 + 641.1x - 60.6x^2$ well ($p < 0.05$), so only the data of whole mungbean seedlings

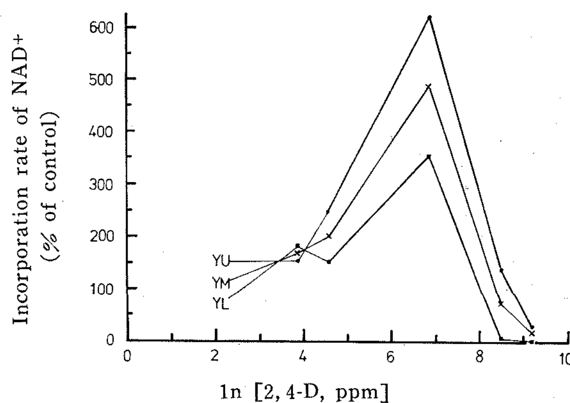

Fig. 1. Effect of 2,4-D concentrations on incorporation rate of NAD+ (% of control). YU=upper part of seedlings; YL=lower part of seedlings; YM =whole seedlings.

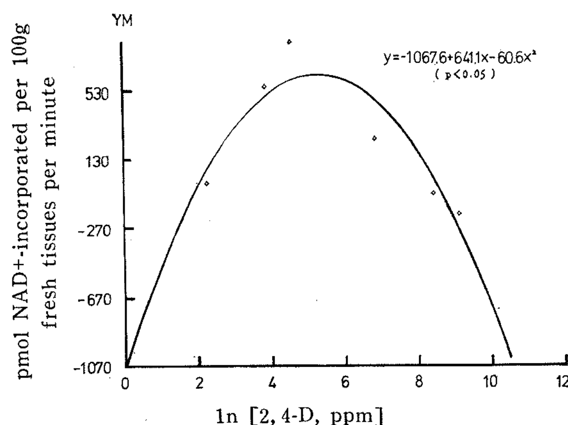

Fig. 2. Relationship between natural logarithm of 2, 4-D concentrations and incorporation rate of NAD+ (rate of 2, 4-D treated samples—controls)

are presented. The curve again had a peak. High concentrations of 2, 4-D seem to inhibit NAD+-incorporation activity of whole seedlings.

Discussion

A conclusion may be drawn from results of Table 1 and related data: hypercotyl is the one with the highest NAD+-incorporating activity among four different tissues examined and root is the lowest. When enzyme activity is expressed as nmol NAD+-incorporated per 100 g fresh tissue, it seems that leaf has the highest incorporating activity, this is due to the fact that leaf contains higher amounts of both protein and DNA than hypercotyl. So it is quite possible that NAD+-incorporating activity is closely related to physiological and biochemical activities of mungbean seedlings. This proposal is further supported by results presented in Table 2. After treatment of 2, 4-D, the enzyme activities of stored crude chromatin of both hypercotyl and hypocotyl change dramatically: hypercotyl downward, while hypocotyl upward.

We have noticed that total incorporations (or specific activity of incorporation) of NAD+ varied to some extent when data of comparable sets of experiments using different batches of rice grain or different chromatin preparations of the same batch of rice were examined (Lin and Kuo, 1981). In this report we have found the same phenomenon for mungbean and the variation is even greater than the rice (Table 5). This may be due to five possible factors: variation of percentage of germination; variation of water content of individual seedlings; effect of plant hormones produced during seed storage; and/or contamination of crude chromatin by cytoplasmic material to various extents; physiological and biochemical states of the isolated chromatin. This needs further examinations.

The upper parts of mungbean seedlings used in both Table 5 and 6 include cotyledons containing high contents of protein and DNA which might lower the apparant specific enzyme activity.

During the calculation of Table 5 and Table 6, we found that for crude enzyme preparations it is more suitable to use NAD^+-incorporated per 100 g fresh tissue than NAD^+-incorporated per mg protein or mg DNA when examining effect of 2,4-D. This suggests that cellular factors other than amounts of protein and DNA may affect NAD^+-incorporating activity.

Considering the pronounced effect of 2,4-D on physiological and biochemical processes of young tissue (hook) and matured portions of hypocotyl of etiolated soybean seedlings, NAD^+-incorporating activity may be involved in important physiological and biochemical processes of etiolated mungbean seedlings and possibly other plants as well since it is under the influence of 2,4-D.

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二氯苯氧乙酸 (2,4-D) 對綠豆芽不同部位之 NAD+ 聚合活性的影響

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從白化的綠豆芽，經過或未經二氯苯氧乙酸 (2, 4-D) 處理，所製得之粗染色質或經 1.7 摩耳 (molar) 蔗糖液離心純化之染色質經貯藏 2 天後測其 NAD+ 聚合活性並以三種不同方式表示。如果活性以聚合之 NAD+ 量 (10^{-9} 摩耳)/毫克蛋白質表示，則上胚軸>葉>下胚軸>根；如果以聚合之 NAD+ 量 (10^{-9} 摩耳)/100 克新鮮組織表示，則葉>上胚軸>下胚軸，根；如果以聚合之 NAD+ 量 (10^{-9} 摩耳)/毫克去氧核糖核酸表示，則上胚軸 ≫ 下胚軸，葉 > 根。二氯苯氧乙酸 (552 ppm, pH 6.6) 對上胚軸及下胚軸的酵素活性影響很顯著而且不同。二氯苯氧乙酸的處理減少上胚軸粗染色質之酵素活性，但是却增加下胚軸之酵素活性。

對通常試料而言，NAD+ 聚合活性之變方分析顯示；(1)綠豆芽之上部 (從子葉下 0.5 公分算起一直往上，含子葉及初生葉) 和下部 (從子葉下 1.0 分分算起往下直到根部) 間無差異存在；(2)同一品種但是在不同次實驗發芽之綠豆芽 (試料) 雖然都在發芽第 7 天收穫但有顯著差異存在；(3)上、下部與試料間之交感作用也存在。對 2,4-D 處理過之試料而言；(1)上、下兩部份間有極顯著差異存在；(2) 2,4-D 之濃度間有極顯著差異存在；(3)上、下部與濃度間無交感效應存在。當整株綠豆芽之 NAD+ 聚合速率 (經 2,4-D 處理過之試料所測得之值扣掉未處理試料之值) 對 2,4-D 濃度 (ppm) 之自然對數作圖時可適用二次方程式 $y = -1067.6 + 641.1x - 60.6x^2$，可信程度爲 95%。