ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 52, nr 1: 77-86
1983

Nitrate reductase and acid phosphatase activities as affected by inorganic phosphate in corn roots

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(Received: May 11, 1982)

Abstract

The deficieny of inorganic phosphate in nutrient solution reduces by about 50 per cent NO_3^- absorption in corn seedlings, it decreases both *in vitro* and *in vivo* nitrate reductase (NR) activity, as well the potential and actual NR level and has a very weak effect on NR induction. Acid phosphatases activities increase in corn roots when the plants are grown in nutrient solution without phosphorus. We suggest that inorganic phosphate is required mainly for maintenance of NR activity rather, than for induction *in vivo* of nitrate reductase. It is not excluded that deficiency of inorganic phosphate in root tissue may be partly supplemented as the result of enhanced acid phosphatase activity.

INTRODUCTION

The requirement of inorganic phosphate for NR activity *in vitro* was observed for *Neurospora* (Kinsky and McElroy 1958) and for wheat embryo (Spencer 1959). Hewitt and Nicholas (1964) suggested that NR *in vitro* is activated by inorganic phosphate. Harper and Paulsen (1969) and Sasakava and Yamamoto (1977) studying the effect of deficient nutrients on NR found that deficiency of phosphorus in nutrient solution decreases specific NR activity in wheat leaves and rice shoots.

We examined the effect of inorganic phosphate on *in vivo* nitrate reductase induction and activity as well as the effect of this compound on NO_3^- uptake and level of acid phosphatases in corn roots.

MATERIAL AND METHODS

Seeds of corn (*Zea mays* L. Kb-270) were treated as described previously (Buczek et al. 1981). After 5 days growth of seedlings on distilled water under a light-dark period (16 h light, 10 000 lux) at 25°C during the day, 20°C at night and 48% relative humidity, the seed remnants were removed and the seedlings transferred to the minus N nutrient solution with or without phosphorus. The −N+P solution contained (in mmol×dm^{-3}): K_2SO_4 — 0.8, $CaSO_4 \cdot 2H_2O$ — 1.2, KH_2PO_4 — 1.0, $MgSO_4$ — 0.4 and micronutrients (Buczek et al. 1981). In the −N−P solution 0.5 mmol K_2SO_4 was included instead of potassium phosphate. The pH was adjusted to 6.0 and the solution was aerated continuously. The plants were grown in these solutions for 3 days under light-dark conditions as above. Then they were transferred to the nitrate solution contained (in mmol×dm^{-3}): KNO_3 — 5, $Ca(NO_3)_2$ — 5, KH_2PO_4 — 1, $MgSO_4$ — 1 and micronutrients. In the −P nitrate solution KH_2PO_4 was replaced by K_2SO_4 with maintained constant potassium concentration. Both nitrate solutions were adjusted to pH 6.0 and aerated continuously. The plants were grown in these solutions for 5 days under the conditions described above or for 1 day under continuous light. The roots were harvested for assays of enzyme activities and analysis of inorganic phosphate. For studying NR induction (Table 2) the corn seedlings were grown for 3 days in −N+P or −N−P solution. Then the roots were excised and incubated in media containing 0.05 M KNO_3 with different buffers (see measurement of NR activity *in vivo*).

Extracts of NR were prepared from fresh root tips (excised 4 cm below the tip) (Buczek 1976). Nitrate reductase activity *in vitro* was assayed in the supernatant fraction following 18 000×g centrifugation of the homogenate, by measuring NADH-dependent production of NO_2^- (Hageman and Flesher 1960) with the exception that instead of K-phosphate buffer, the HEPES-buffer pH 7.0 was used. The NR activity was calculated as nmoles NO_2^- formed per gram fresh weight per hour. Three replicate samples were harvested at each time period and the experiment was repeated at least 3 times.

Measurement of NR activity *in vivo:* **Method A** (Jaworski 1971). Root sections (0 to 4 mm from tip) were excised, weighed (250-300 mg) and placed in 25 cm^3 Erlenmayer flasks containing 0.05 M K-phosphate buffer (pH 7.0), 0.1 M KNO_3, 5% v/v propanol in total 5 cm^3 volume and incubated for 1 h in water bath at 32°C in darkness. After incubation, the flasks were cooled in an ice bath, and nitrite released to the medium was determined by the procedure of Sanderson and Cocking (1964). **Method B** was similar to method A except that K-phosphate buffer was replaced by TCGM buffer (pH 7.0) containig 0.012 M Tris. 0.008 M citrate. 0.012 M glycine and 0.012 M maleate

(Bieleski 1974). **Method C** was a modification of the Hageman et al. (1980) procedure. The 2 mM $CaSO_4$ was used instead of phosphate buffer to avoid inclusion of inorganic phosphate. Potential and actual NR activity (Bar-Akiva et al. 1970) were measured by method C, but in the case of actual NR, KNO_3 was replaced by K_2SO_4 keeping the potassium concentration constant. Nitrate reductase was expressed in nmoles of NO_2^- formed per 1 g fresh weight per hour. Each value in the figures or tables represents a mean of 6 replications and each experiment was repeated at least three times.

Extracts of acid phosphatases were prepared from fresh corn roots and the 18 000×g supernatant fraction was used for assay of acid phosphatases activity according to the method described in the preceeding paper (Buczek et al. 1981). The enzyme activity was expressed in μmoles of P_i liberated from para-nitrophenyl phosphate (p-NPP) per gram fresh weight per hour. Inorganic phosphate (P_i) was determined colorimetrically by the Fiske and Subbarow (1925) method.

Nitrate uptake was assayed by measuring the loss of NO_3^- from the nutrient solution (Cataldo et al. 1975). The content of inorganic phosphate in roots was determined in fresh material after extraction with cold 10% trichloroacetic acid, by the method of Fiske and Subbarow (1925) using ammonium molybdate and 1-amino-2-naphthol-4--sulphonic acid sodium sulphite.

All biochemicals were of analytical grade, purchased from Sigma (NADH, p-NPP) and POCH-Gliwice.

RESULTS

Changes of NR activity in roots of corn seedlings growing five days either in the presence or absence of inorganic phosphate in nutrient solution are shown in Fig. 1A. The roots were excised, homogenised and NR activity was assayed *in vitro*. The lack of phosphorus in the nutrient solution caused a decrease of NR activity in roots and its complete disapearance after 4 days.

The lack of inorganic phosphate in the nutrient solution caused an enhanced acid phosphatases activity in the roots, especially at pH 4.5 (Fig. 1B). The content of inorganic phosphate in roots of −P plants was very low in comparison with that in +P ones. However, an almost complete decline of free inorganic phosphate in roots of −P plants was observed after 4 days growth (Fig. 2). As in Fig. 1A, the decline in inorganic phosphate in roots was correlated with decay of NR activity.

The absorption of NO_3^- (Fig. 3) by corn seedlings growing in −P solution was decreased by about 50 per cent in comparison with that

in +P plants. However, the deficiency of P in the nutrient solution did not inhibit completely NO_3^- uptake.

The *in vivo* assays of NR activity in corn roots during induction of enzyme (Fig. 4A) showed a marked reduction of the potential NR in

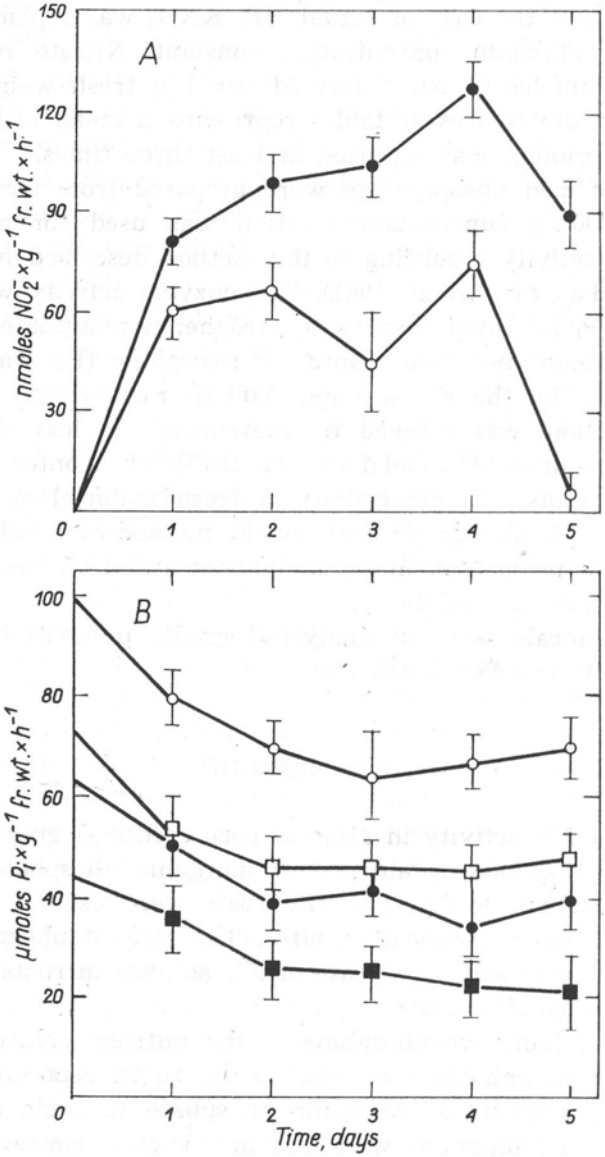

Fig. 1. Nitrate reductase (A) and acid phosphatase (B) activities in corn roots in the presence or absence of inorganic phosphate in nutrient solution
Nitrate reductase (A): plus phosphorus — solid circles; minus phosphorus — open circles; NR activity was assayed *in vitro*. Acid phosphatases (B): plus phosphorus — solid circles (pH 4.5) and solid squares (pH 6.5); minus phosphorus — open circles (pH 4.5) and open squares (pH 6.5). Vertical bars indicate ± SE of mean values of at least 3 determinations

roots growing without phosphorus. A complete lack of P in the nutrient solution caused a lesser diminution of the actual NR level, although the differences were significant. At the same time, the activities of acid phosphatases in roots of plants growing without phospho-

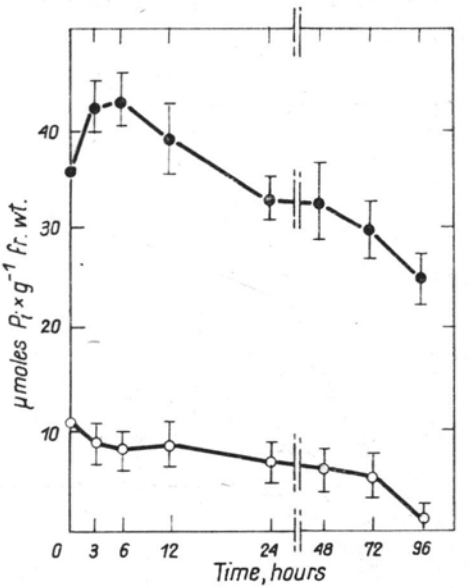

Fig. 2. Content of inorganic phosphate in corn roots
Plus phosphate — solid circles; minus phosphate — open circles. Vertical bars represent ± SE

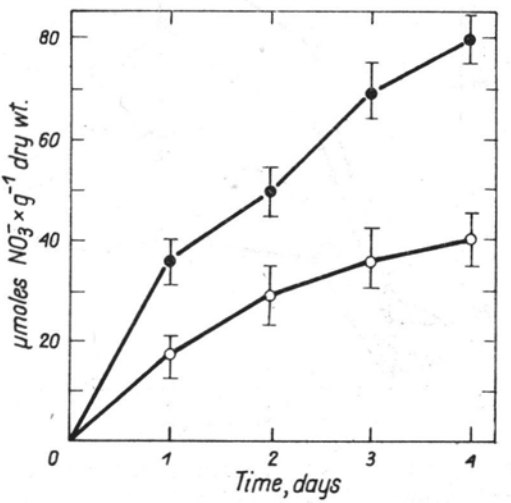

Fig. 3. Uptake of NO_3^- by corn seedlings growing in solution with or without inorganic phosphate
Plus phosphorus — solid circles; minus phosphorus — open circles. Data represent mean ± SE of 6 replicates

rus were relatively high in comparison with those in plants growing in solution containing phosphorus. The increase of acid phosphatases was found to occur after 6 h growth of plants in nitrate solution containing no inorganic phosphate (Fig. 4B), while the content of inorganic phosphate in roots during 24-h growth of plants in —P solution was indeed low but constant (Fig. 2).

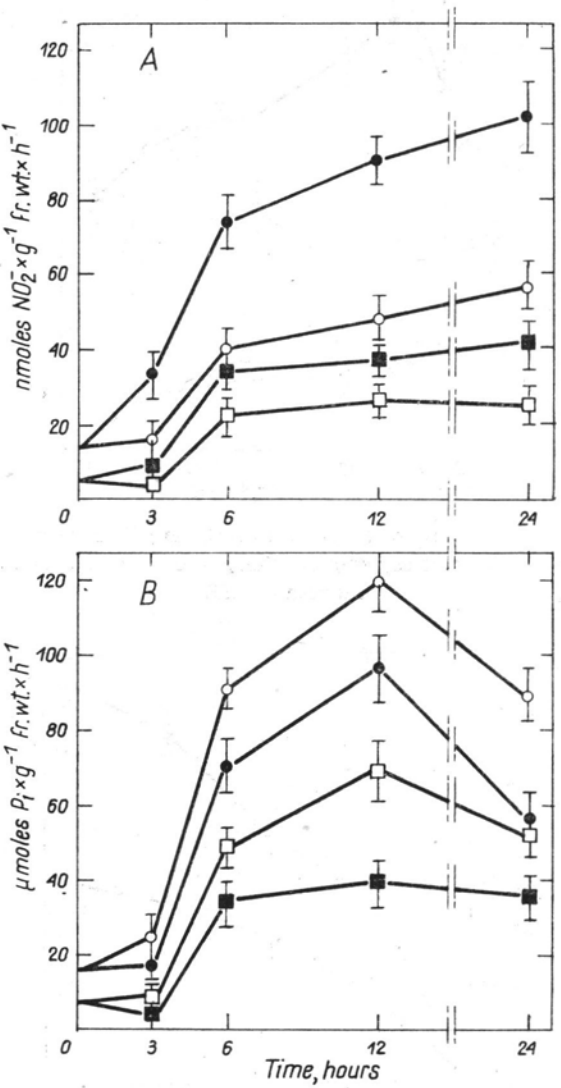

Fig. 4. Potential and actual nitrate reductase (A) and acid phosphatase (B) activities in corn roots growing in solutions with or without inorganic phosphate Nitrate reductase (A) was assayed *in vivo* (Method C): plus phosphorus — solid circles (potential activity) and solid squares (actual activity); minus phosphorus — open circles (potential activity) and open squares (actual activity). Acid phosphatases (B): explanation as for Fig. 1

Potential and actual NR level (Table 1) were assayed in excised root tips of plants growing previously for 24 h in nitrate containing solution with or without P. NR activity was assayed *in vivo* by method C (see Materials and Methods). The data shows that potential NR activity in roots of plants growing without P was more decreased than the actual one, although the actual NR level in —P plants was significantly reduced after two hours of incubation.

Table 1

Effect of phosphorus on "potential" and "actual" nitrate reductase (nmoles $NO_2^- \times g^{-1}$ fresh weight $\times h^{-1}$) in corn roots

Nutrient solution	Nitrate reductase	Time of assay, h		
		1	2	3
+P	potential	118.37±14.95 a	231.33±40.00 c	241.85±19.60 c
—P	potential	41.92± 6.29 b	141.25±26.65 d	173.02±17.36 f
+P	actual	31.99± 7.41 b	90.45±11.74 a	101.55±10.12 a
—P	actual	31.50± 5.11 b	72.94±12.82 e	87.56± 9.37 e

NR activity was assayed *in vivo* by method C. Mean values with similar letters did not differ significantly at 5% level. Each mean represents the average of 6 replications.

The effect of phosphorus on *in vivo* induction of NR is shown in Table 2. The data show that inorganic phosphate is not necessary for activation (induction) of nitrate reductase *in vivo*, although NR activity was lowest when the roots were incubated in medium containig no phosphate. It was found however, that the presence of inorganic phosphate in the ambient solution is necessary for maintenance of NR activity on a relatively high level.

Table 2

Effect of phosphorus on *in vivo* induction of NR (nmoles $NO_2^- \times g^{-1}$ fresh weight $\times h^{-1}$) in corn root tips excised from plants growing 3 days in minus N solution with or without inorganic phosphate

Nutrient solution	Incubation medium	Time of assay, h		
		4	6	8
+P	phosphate buffer	14.60±1.32 a	144.20±12.13 c	286.12±15.32 d
—P		14.07±1.42 a	119.30±10.40 d	282.07±13.12 d
+P	$CaSO_4$	8.91±1.42 b	21.30± 1.81 e	97.71± 5.14 f
—P		9.72±0.97 b	14.31± 0.97 a	18.92± 1.01 c
—P	phosphate buffer	9.86±1.32 b	41.04± 2.02 g	259.80±24.12 d
—P	TCGM buffer	10.50±1.21 b	18.70± 1.43 e	50.74± 3.18 g

Each mean represents the average of 6 replications.

DISCUSSION

The dependence of NR activity on inorganic phosphate was at first observed by Spencer (1959) who found that the presence of inorganic phosphate in incubation medium was required for maximum NR activity in wheat germs. Similarily Harper and Paulsen (1969) and Sasakawa and Yamamoto (1977) found in wheat and rice seedlings growing in medium with deficient phosphorus a considerable decrease of specific NR activity and of nitrate content in plants. Our results showed that deficiency of phosphorus in the nutrient solution caused a strong decrease of NR activity, both *in vitro* and *in vivo* and reduction of nitrate uptake. Furthermore the lack of phosphorus in the nutrient solution caused an increase of acid phosphatases activities, while the level of inorganic phosphate in roots of –P plants was in fact very low but constant.

The simplest explanation consistent with our observation is that the decrease of nitrate absorption in the absence of inorganic phosphate, limits the level of NR activity. It seems also possible to assume that NO_3^- and HPO_4^{2-} are correlatively absorbed. Gericke (1943) found that higher plants require an increased supply of phosphorus when nitrate is being utilised. Recently Thien and McFee (1972), Taber and McFee (1972) and Barta (1977) have shown that NO_3^- pretreatment stimulates the HPO_4^{2-} uptake.

It seems that phosphorus deficiency has direct effect on induction or activation of nitrate reductase. However, the fact that the lack of inorganic phosphate in both nutrient solution and incubation medium decreased not only the potential but also the actual NR level, may suggest that inorganic phosphate is required not only for maintenance of NR activity, but also for its activation. Some evidence of this view has been given by Kinsky and McElroy (1958) and Hewitt and Nicholas (1964). These authors suggested the requirement of inorganic phosphate for activation of hexavalent molybdenum in molybdoflavoprotein. However, this supposition has not been confirmed.

It appears that the deficency in inorganic phosphate in roots of –P plants may be partly and perhaps for a short time supplemented by an increment of acid phosphatases. It appears that our results confirm this supposition because the level of acid phosphatases increased markedly (Fig. 1B) and the level of inorganic phosphate, although very low, was almost constant (Fig. 2). It is not excluded that this fact finds reflection in the relatively low diminution of actual NR activity in –P roots in comparison with the potential one. However, when the activity of NR is high, as in the case of potential NR, the deficiency of inorganic phosphate cannot be compensated by the action of phosphatase. Thus, the increase in acid phosphatase found in our experiments results

perhaps from the well known fact that a lack of P in the medium may stimulate a number of acid phosphatases in plants (Kubicz 1973). It seems, thus, that there is a very weak correlation between NR and acid phosphatases in corn roots.

REFERENCES

Bar-Akiva A., Sagiv J., Leshem J., 1970. Nitrate reductase activity as an indicator for assessing the nitrogen requirement of grass crops. J. Sci. Agric. 21: 405-407.

Barta A. L., 1977. Uptake and transport of calcium and phosphorus in *Lolium perenne* in response to N supplied to halves of a divided root system. Physiol. Plant. 39: 211-124.

Bieleski R. L., 1974. Development of an externally-located alkaline phosphatase as a response to phosphorus deficiency. In: Mechanisms of Regulation of plant growth. Bieleski R. L., Ferguson A. R. and Cresswell M. M. (eds.), Bull. 12 Roy, Soc. New Zeland, Wellington, pp. 165-170.

Buczek J., 1976. The role of light in the induction of nitrate reductase and nitrite reductase in cucumber seedlings. Acta Soc. Bot. Pol. 45: 77-92.

Buczek J., Burzyński M., Suder-Moraw A., 1981. Effect of calcium and magnesium ions on phosphatases from *Zea mays* L. roots. Acta Physiol. Plant. 3: 13-22.

Cataldo D. A., Haroon M., Schrader L. E., Young V. L., 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicilic acid. Comm. Soil Sci. 6: 71-80.

Fiske C. H., Subbarow Y., 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400.

Gericke S., 1943. Die Dingwirkung der Phosphorsäure bai verschidener Stickstoffnährung der Pflance. Bodenkunde u. Pflanzenernährung 33: 196-215.

Hageman R. H., Flesher D., 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 34: 700-708.

Hageman R. H., Reed A. J. Femmer R. A., Sherrard J. H., Dalling M. J., 1980. Some new aspects of the *in vivo* assay for nitrate reductase in wheat (*Triticum aestivum* L.) leaves. I. Reevaluation of nitrate pool sizes. Plant Physiol. 65: 27-32.

Harper J. E., Paulsen G. M., 1969. Nitrogen assimilation and protein synthesis in Wheat seedlings as affected by mineral nutrition. I. Macronutrients. Plant Physiol. 44: 69-74.

Hewitt E. J., Nicholas D. J. D., 1964. In: Modern methods of plant analysis. Linskens H. F., Sanwal B. D. and Tracey M. V. (eds.), 7: 67-172.

Jaworski E. G., 1971. Nitrate reductase assay in intact plant tissues. Biochem. Biophys. Res. Commun. 43: 1274-1279.

Kinsky S. C., McElroy W. D., 1958. *Neurospora* nitrate reductase, the role of phosphate, flavin and cytochrome c reductase. Arch. Biochem. Biophys. 73: 466-473.

Kubicz A., 1973. Acid phosphatases, their heterogenity, structure and properties. Postępy Biochemii 19: 37-54.

Sanderson G. W., Cocking E. C., 1964. Enzymatic assimilation of nitrate in tomato plants. I. Reduction of nitrate to nitrite. Plant Physiol. 39: 416-422.

Spencer D., 1959. A DPNH-specific nitrate reductase from germinating wheat. Austr. J. Biol. Sci. 12: 181-196.
Sasakawa H., Yamamoto Y., 1977. Nitrate assimilation in rice seedlings as affected by mineral nutrition. Plant Cell Physiol. 18: 215-224.
Taber H. G., McFee W. W., 1972. Nitrogen influence on phosphorus uptake by *Pinus radiata* seedlings. Forest Sci. 18: 126-132.
Thien S. J., McFee W. W., 1972. Effect of nitrogen on phosphorus transport systems in *Zea mays* L. Soil Sci. Am. Proc. 34: 87-90.

Aktywność reduktazy azotanowej i kwaśnych fosfataz w korzeniach kukurydzy w zależności od obecności lub braku nieorganicznego fosforanu w pożywce

Streszczenie

Badano wpływ braku fosforu w pożywce na poziom aktywności reduktazy azotanowej (NR) i kwaśnych fosfataz oraz na pobieranie NO_3^- i zawartość nieorganicznych fosforanów w korzeniach kukurydzy. Stwierdzono, że brak fosforu w pożywce zahamował w 50 procentach pobieranie azotanów oraz obniżył aktywność NR mierzoną zarówno *in vitro* jak *in vivo*. Brak fosforu wpływał przede wszystkim na obniżenie tzw. „potencjalnej" aktywności NR i w mniejszym stopniu, aczkolwiek istotnie, na tzw. „aktualną" aktywność enzymu. Aktywność badanych kwaśnych fosfataz zwiększała się w korzeniach roślin rosnących przy niedostatku fosforu w pożywce. Wyniki sugerują, że nieorganiczne fosforany niezbędne są głównie do utrzymania aktywności NR na względnie wysokim poziomie, a w mniejszym stopniu wpływają na indukcję, względnie aktywację NR. Nie jest wykluczone, że podwyższona aktywność kwaśnych fosfataz spowodowana brakiem fosforu w pożywce uzupełnia, przynajmniej częściowo, w tkankach niedostatek nieorganicznych fosforanów.