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Effect of Hexachlorobenzene on Haem Synthesis

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Several drugs are known to induce the liver microsomal mixed-function oxidase system when administered *in vivo* or even *in vitro* in cell culture. A sequence of events has been suggested in which the drug is visualized to induce δ-aminolaevulinate synthetase, the first and rate-limiting enzyme of the haem-biosynthetic pathway, which is followed by enhanced haem synthesis and cytochrome *P*-450 content, facilitating the increase in the drug-metabolizing activity of the liver microsomal fraction. The present studies show that the fungicide hexachlorobenzene, when administered to female rats, can lead to enhanced amounts and rate of synthesis of cytochrome *P*-450 under conditions when the rate of total haem synthesis has not appreciably altered. The subsequent increase in the rate of total haem synthesis as well as the initial increase in amounts of cytochrome *P*-450 are brought about under conditions when δ-aminolaevulinate synthetase activity remains constant. However, manifestation of porphyria due to prolonged drug administration is accompanied by a twofold increase in δ-aminolaevulinate synthetase activity. The increase in enzyme activity appears to be due to a decreased degradation rate of the enzyme.

A wide variety of drugs are metabolized by the mixed-function oxidase system of the liver microsomal fraction (referred to below as microsomes) (Conney, 1967; Kuntzman, 1969). It has been observed for several drugs that soon after their administration to experimental animals there is an increase in the liver microsomal cytochrome *P*-450, one of the components of the drug-metabolizing system. Increased cytochrome *P*-450 has been associated with an increased rate of haem synthesis. A sequence of events has been suggested in which the drug is visualized to induce δ-aminolaevulinate synthetase, the first and rate-limiting enzyme of the haem-biosynthetic pathway, which is followed by enhanced haem synthesis and cytochrome *P*-450 content, facilitating the increase in the drug-metabolizing activity of the liver microsomes (Granick, 1966; Schmid *et al.*, 1966; Baron & Tephly, 1970).

Hexachlorobenzene is a fungicide and has been shown to be associated with the induction of porphyria in human beings and experimental animals (Ockner & Schmid, 1961; De Matteis *et al.*, 1961). The present study with hexachlorobenzene has been carried out to examine the relationship between δ-aminolaevulinate synthetase induction and accelerated haem synthesis as exemplified by enhanced amounts of cytochrome *P*-450 and manifestation of porphyria.

Materials and Methods

Materials

Chemicals. Hexachlorobenzene (technical grade) was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. It was recrystallized twice before use.

Animals. Female rats (90–100g) of the local Institute strain were fed *ad libitum* a stock diet containing 0.2% hexachlorobenzene. The animals were killed at different time-intervals by decapitation and the livers were processed for various constituents.

Processing of liver

Pooled livers were homogenized in 4vol. of 1.15% (w/v) KCl and portions of the homogenates were used for the assay of δ-aminolaevulinate synthetase, δ-aminolaevulinate dehydratase and estimation of amounts of δ-aminolaevulinate, porphobilinogen and porphyrin. The remaining portion of the homogenate was centrifuged at 15000*g* for 15min and the postmitochondrial supernatant was spun at 100000*g* for 60min to isolate the microsomes. The microsomal pellet was resuspended in KCl and centrifuged at 125000*g* for 60min. The pellet was rinsed once with KCl and then suspended in 0.05M-potassium phosphate buffer, pH7.5. This suspension was used for the determination of cytochrome *P*-450.

Measurement of liver δ-aminolaevulinate, porphobilinogen and porphyrins

Portions of the liver homogenates were deproteinized with trichloroacetic acid. The supernatants were adjusted to pH4.5–5.0 and δ-aminolaevulinate and porphobilinogen were fractionated by using Dowex-1 (acetate form) columns and acetylacetone as described by Marver *et al.* (1966). Portions of the effluents containing porphobilinogen and δ-aminolaevulinate pyrrole were treated with equal volumes of Ehrlich reagent and the colour was measured at 556nm (Mauzerall & Granick, 1956). Porphyrins

were determined in portions of the liver homogenate with ethyl acetate–acetic acid (3:1, v/v) as the extracting solvent, followed by HCl fractionation as described by Sardesai *et al.* (1964). The only modification introduced was in the measurement of uroporphyrin, where the sodium acetate washings of the total porphyrin extract were processed with acetic acid–pentanol (1:10, v/v) as the extractant, as described by With (1968).

Assay of δ-aminolaevulinate synthetase and δ-aminolaevulinate dehydratase

δ-Aminolaevulinate synthetase was assayed in liver homogenates by the procedure described by Marver *et al.* (1966). The incubation mixture contained, in a total volume of 2ml: 1.0ml of homogenate, 200μmol of glycine, 20μmol of EDTA and 150μmol of tris–HCl buffer, pH 7.2. The incubation was carried out at 37°C for 60min. The reaction was stopped by the addition of 0.5ml of 25% trichloroacetic acid. δ-Aminolaevulinate was estimated directly in the supernatant or after column fractionation as described earlier.

δ-Aminolaevulinate dehydratase was assayed in the homogenates as described by Gibson *et al.* (1956). The homogenate, in the presence of 0.05M-potassium phosphate buffer, pH 6.8, was preincubated with glutathione (10μmol) for 30min at 37°C. The total volume of the reaction mixture was 2ml. After incubation for 60min the reaction was stopped with 1ml of 12.5% trichloroacetic acid containing 0.02M-$HgCl_2$. After centrifugation a portion of the supernatant was used to estimate content of porphobilinogen.

Cytochrome P-450 estimation

This was done by measuring the CO-difference spectrum of dithionite-reduced microsomal suspensions in a Cary 14 spectrophotometer. The cytochrome *P*-450 concentration was calculated with the use of an extinction coefficient of $91\,mM^{-1}\cdot cm^{-1}$ for E_{450}–E_{490} (Omura & Sato, 1964).

Incorporation of amino acids

[2-^{14}C]Glycine and δ-amino[4-^{14}C]laevulinate incorporation into total and microsomal haem. Animals fed on 0.2% hexachlorobenzene as well as normal diets were given an intraperitoneal injection of [2-^{14}C]glycine (5μCi/animal) or δ-amino[4-^{14}C]-laevulinate (2μCi/animal); 30min and 60min after the injection the animals were killed and the livers isolated after perfusion with saline. Liver homogenates were prepared by using KCl as described above and a portion of the homogenate was kept to measure uptake of ^{14}C radioactivity and the rate of total haem synthesis. The microsomal pellet was treated as described by Omura & Sato (1964) to separate the cytochromes b_5 and *P*-450. Briefly, the microsomal suspension was incubated in a nitrogen atmosphere with steapsin, final concn. 0.14% (w/v), at 37°C for 60min to solubilize cytochrome b_5. After the incubation, the suspension was centrifuged at 125000*g* for 90min and the pellet was used as a source of cytochrome b_5-free cytochrome *P*-450. Haem was isolated from the cytochrome *P*-450 fraction and radioactivity determined by methods described by Baron & Tephly (1970) and Beattie (1971). Briefly, the microsomal suspension was successively treated with cold acetone, methanol–chloroform (2:1, v/v) and again with acetone to remove the lipids. The residue was extracted with cold acid–acetone (1.5ml of 10M-HCl in 100ml of acetone) to split the haem from the haemoprotein. The extract was diluted with water and the haem was extracted into peroxide-free ether. The ether layer was washed once with 5% NaCl and once with water. It was then divided into two equal portions. One portion was quantitatively transferred to filter-paper discs, air-dried and used for radioactivity measurements. The other portion was evaporated to dryness under a stream of nitrogen and the haem content was measured as the pyridine haemochromogen. Chromatography of the ether layer also revealed that the entire radioactivity derived from [2-^{14}C]glycine or δ-amino[4-^{14}C]laevulinate is associated with the haem spot. Other workers have also shown that the radioactivity in the haem isolated from the cytochrome *P*-450 fraction prepared as described above is not contaminated with non-haem material (Levin & Kuntzman, 1969; Greim *et al.*, 1970; Meyer & Marver, 1971). For measuring the rate of total haem synthesis, 1ml of blood was added as carrier to portions of the liver homogenate and haemin was isolated by the procedure described by Satyanarayana Rao *et al.* (1971). The twice-recrystallized haemin was solubilized and extracted into peroxide-free ether. The washed ether layer was used for radioactivity measurements and determination of haem content as described above.

Total radioactivity uptake by liver was measured after suitably diluting a portion of the homogenate with deoxycholate (1%, w/v, final concn.).

Amino acid incorporation into microsomes in vitro. This was studied essentially by the procedure described by Munro *et al.* (1964). Microsomes were isolated by layering 22ml of the postmitochondrial supernatant over 10ml of 1M-sucrose and centrifuging at 100000*g* for 2h. The pH 5 fraction was prepared from the postmicrosomal supernatant of normal animals. The supernatant was passed through a Sephadex G-25 column and then adjusted to pH 5.0 with 1M-acetic acid. The precipitate was centrifuged off and the supernatant was adjusted to pH 7.5 and then subjected to ammonium sulphate fractionation.

The 0–0.65-saturated fraction was collected and dialysed. This was mixed with the pH 5 precipitate, constituting the pH 5 fraction. The microsomal suspension was incubated at 37°C in the presence of amino acid mixture (leucine omitted), pH 5 fraction, energy-generating system and [U-^{14}C]leucine for different intervals of time. The reaction was stopped with 5% (w/v) trichloroacetic acid containing unlabelled leucine. The precipitate was washed with cold trichloroacetic acid, ethanol–ether, hot trichloroacetic acid and ether. The final residue was dissolved in formic acid and portions were used for measurement of radioactivity.

RNA was determined in microsomal suspensions by the procedure of Munro & Fleck (1966).

Radioactivity measurements

The preparations were all added to filter-paper discs as planchets and these were placed in vials containing 10ml of 0.5% (w/v) 2,5-diphenyloxazole in toluene. The radioactivity measurements were made in a liquid-scintillation counter (Beckman LS 100).

Half-life determination of δ-aminolaevulinate synthetase

Animals fed with the hexachlorobenzene diet or those treated with injections of allylisopropylacetamide (40mg/100g) were injected with cycloheximide (1mg/100g) and then killed at different intervals of time. The cycloheximide injection was repeated every 45min until the animals were killed. δ-Aminolaevulinate synthetase was assayed in liver homogenates as described above.

Protein determination

This was done by the method of Lowry *et al.* (1951) with bovine serum albumin used as the standard.

Results and Discussion

The results given in Table 1 indicate that female rats fed on a diet containing 0.2% hexachlorobenzene show evidences of porphyria after only 2 weeks of intake of hexachlorobenzene. Elevated amounts of δ-aminolaevulinate as well as uro- and copro-porphyrin are detectable only around this period. Elevated amounts of porphobilinogen are seen only around the fourth week of hexachlorobenzene treatment. De Matteis *et al.* (1961) also observed a similar picture of an increase in copro- and uro-porphyrin with a terminal excretion of a considerable amount of porphobilinogen. However, Table 1 also indicates that cytochrome *P*-450 tends to increase in amount as early as 24h after drug intake and significant increases are seen after 48h. The amount of cytochrome *P*-450 remains constant after the first week on hexachlorobenzene diet during the rest of the treatment period.

An increase in δ-aminolaevulinate synthetase is always associated with an enhanced rate of haem synthesis and it was of interest to examine the activity of this enzyme under conditions of feeding with hexachlorobenzene. The results presented in Table 2 indicate that no change in δ-aminolaevulinate synthetase activity could be detected, at least during the first 72h of drug treatment when cytochrome *P*-450 shows a striking increase in amount. However, a twofold increase in δ-aminolaevulinate synthetase

Table 1. *Effect of feeding with hexachlorobenzene on the amounts of porphyrin precursors, porphyrins and cytochrome P-450 in the liver of female rats*

The experimental details are given in the text. *P* was calculated with respect to the untreated control from the mean±S.D. obtained from four experiments. The other values represent an average of two experiments and two livers were pooled in each experiment. n.d., Not detected.

Period of treatment (days)	Liver content (μg/g wet wt.)				Liver cytochrome *P*-450	
	δ-Amino-laevulinate	Porphobilinogen	Uroporphyrin	Coproporphyrin	(nmol/mg of microsomal protein)	*P*
0	0.9	n.d.	n.d.	n.d.	0.71±0.10	—
1	1.0	n.d.	n.d.	n.d.	0.89±0.05	<0.05
2	1.1	n.d.	n.d.	n.d.	1.21±0.11	<0.02
3	1.1	n.d.	n.d.	n.d.	1.62±0.13	<0.01
7	2.5	n.d.	0.52	1.20	1.91±0.15	<0.01
15	3.2	1.1	6.92	1.86	2.01	
22	3.3	3.2	7.38	1.26	1.96	
29	3.1	3.3	8.10	1.52	1.75	

Table 2. *Effect of feeding with hexachlorobenzene on the activities of δ-aminolaevulinate synthetase and δ-aminolaevulinate dehydratase in the liver of female rats*

The experimental details are given in the text. *P* was calculated with respect to the untreated control from the mean±S.D. obtained from four experiments. δ-Aminolaevulinate dehydratase values represent an average of two experiments and two livers were pooled in each experiment.

Period of treatment (days)	δ-Aminolaevulinate synthetase (nmol of δ-aminolaevulinate/g of liver)	*P*	δ-Aminolaevulinate dehydratase (μmol of porphobilinogen/g of liver)
0	16.1±2.1		0.35
1	15.1±1.8	>0.05	0.31
2	14.5±1.6	>0.05	0.32
3	13.2±3.5	>0.05	0.36
7	18.9±2.5	>0.05	0.40
15	33.3±4.3	<0.01	0.28
22	32.1±2.7	<0.01	0.25
29	27.2±2.1	<0.01	0.18

activity was always detected at the time of manifestations of porphyria. Wada *et al.* (1968) detected a twofold increase in δ-aminolaevulinate synthetase activity within 24h of giving hexachlorobenzene to mice at 2.5% (w/v) in the diet. However, at this dosage of the drug increase in amounts of cytochrome *P*-450 as well as manifestations of porphyria were apparent within 24h. In the present investigation, administration of hexachlorobenzene at 0.2% (w/w) in the diet to female rats permits the resolution of the two events, namely the increase in cytochrome *P*-450 content and the manifestation of porphyria. δ-Aminolaevulinate dehydratase activity actually decreases with prolonged drug treatment. This is possibly due to the feed-back inhibition of the enzyme by the accumulated porphyrins (Satyanarayana Rao *et al.*, 1970). In spite of the decrease in the enzyme activity, porphobilinogen tends to accumulate, probably because of a block in the conversion of uroporphyrin into coproporphyrin and because δ-aminolaevulinate dehydratase is present in considerable excess of δ-aminolaevulinate synthetase even after the manifestation of inhibition.

It is of interest to examine whether the rate of haem synthesis has altered under conditions when there is an increase in amount of cytochrome *P*-450 but no change in δ-aminolaevulinate synthetase activity. The results obtained with [2-^{14}C]glycine (Table 3) show that, after 24h of hexachlorobenzene intake in the diet, there is a 10% increase in the rate of total haem synthesis and even this increase is not statistically significant. The increase in the specific radioactivity of cytochrome *P*-450 haem is nearly 100%. After 72h the rate of total haem synthesis increases by almost 50%, but that of cytochrome *P*-450 haem decreases to a value similar to that of the controls. The decrease in the specific radioactivity of cytochrome *P*-450 haem at the 72h period is evidently due to the dilution of the label by the unlabelled haemoprotein, which has accumulated in considerable amounts by this time.

Essentially a similar picture is observed with δ-amino[4-^{14}C]laevulinate as the precursor (Table 4) except that the increase in total haem synthesis is not evident at 24h of feeding with hexachlorobenzene and is marginal at 72h. Landaw *et al.* (1970) have also observed that an increased turnover of bilirubin due to phenobarbital administration is demonstrable only with [2-^{14}C]glycine and not with δ-amino[4-^{14}C]laevulinate, because of the greater dilution of the tracer δ-aminolaevulinate by endogenous δ-aminolaevulinate.

Two features of the results presented in Tables 3 and 4 merit discussion. First, at the start the rate of cytochrome *P*-450 haem synthesis is considerably more than that of total haem synthesis. The results obtained with δ-amino[4-^{14}C]laevulinate particularly emphasize the point that even under conditions when an enhanced rate of total haem synthesis is not discernible, a significant channelling of the available haem towards cytochrome *P*-450 is taking place. The drain in the available haem is possibly compensated for subsequently by an increase in the rate of total haem synthesis, as is observed at 72h with [2-^{14}C]glycine (Table 3). Two recent reports have indicated conditions where amounts of cytochrome *P*-450 increase without any change in δ-aminolaevulinate synthetase activity: in neonatal rats administration of phenobarbital leads to an increase in amounts of cytochrome *P*-450 without affecting δ-aminolaevulinate synthetase activity (Song *et al.*, 1971); Bock *et al.* (1971) have reported that, in fed adult rats, pheno-

Table 3. *Effect of feeding with hexachlorobenzene on* [2-^{14}C]*glycine incorporation into total haem and cytochrome P-450 haem*

Animals fed on the hexachlorobenzene diet as well as controls were given a 60min pulse of [2-^{14}C]glycine intraperitoneally (5μCi/animal). Livers from two animals were pooled and processed as described in the text. Blood (1ml) was added as carrier to 4ml of liver homogenate for isolation of total haem. The values represent the means±S.D. from three experiments. *P* was calculated with respect to the untreated control.

Period of treatment (days)	^{14}C radioactivity: $10^5\times$Total uptake (c.p.m./g of liver)	Total haem (c.p.m./μmol of haemin)	*P*	Cytochrome *P*-450 haem (c.p.m./nmol of haemin)	*P*
0	2.21	228±15	—	1122±46	—
1	2.35	255±22	>0.10	2216±98	<0.01
3	2.52	346±19	<0.05	1059±57	>0.10

Table 4. *Effect of feeding with hexachlorobenzene on δ-amino*[4-^{14}C]*laevulinate incorporation into total haem and cytochrome P-450 haem*

Animals fed on the hexachlorobenzene diet as well as controls were given a pulse of δ-amino[4-^{14}C]laevulinate intraperitoneally (2μCi/animal). The animals were killed at 30 and 60min after the tracer administration. Livers from two animals were pooled and processed as described in the text. Blood (1ml) was added as carrier to 4ml of liver homogenate for isolation of total haem. Each value represents an average of two experiments.

Period of treatment (days)	^{14}C radioactivity: $10^4\times$Total uptake (c.p.m./g of liver)		Total haem (c.p.m./μmol of haemin)		Cytochrome *P*-450 haemin (c.p.m./nmol of haemin)	
Time (min) ...	30	60	30	60	30	60
0	0.92	1.37	621	1356	7346	8462
1	0.83	1.56	615	1312	11262	12122
3	0.96	1.65	656	1372	8561	8126

barbital brings about a similar effect although in starved animals an increase in amounts of cytochrome *P*-450 is accompanied by δ-aminolaevulinate synthetase induction.

Thus a primary effect of hexachlorobenzene could be to enhance the rate of apocytochrome *P*-450 synthesis rather than that of haem synthesis. The results presented in Table 5 show that feeding with hexachlorobenzene results in enhanced microsomal protein content. Isolated microsomes from drug-treated animals also show an enhanced capacity for protein synthesis *in vitro* (Fig. 1). These results, in conjunction with the increased cytochrome *P*-450 contents at early stages of drug treatment, emphasize the possibility that the increase in the rate of apocytochrome *P*-450 synthesis could be the primary effect. Attempts to study the rate of apocytochrome *P*-450 synthesis can be made only after the problems regarding its solubilization, stabilization and purification to a homogeneous state are resolved. Recent studies have made this possibility brighter (Lu & Coon, 1968; Lu *et al.*, 1971; Mitani *et al.*, 1971).

The second feature of the results presented in Table 3 is that despite a 50% increase in the rate of total haem synthesis after 72h on hexachlorobenzene diet, there is no change in δ-aminolaevulinate synthetase activity. On the basis that δ-aminolaevulinate synthetase is the only rate-limiting enzyme of the haem-biosynthetic pathway (Granick, 1966), these results can be explained by assuming that at least during the initial stages of drug treatment there is an efficient utilization of haem for haemoprotein synthesis. Since the rate of total haem synthesis measured in the present experiments, as well as those reported in the literature, reflects almost entirely that present bound to haemoproteins, any effect of the drug in minimizing the loss of haem towards breakdown can lead to enhanced labelling of the haem moieties of haemoproteins. Although a free haem pool can be visualized, from which haem is utilized

Table 5. *Effect of feeding with hexachlorobenzene on liver weight and microsomal protein content*

The experimental details are given in the text. The values represent the means±S.D. from four experiments and two livers were pooled in the determination of microsomal protein content.

Period of treatment (days)	Body wt. (g)	Liver wt. (g)	Microsomal protein content (mg/g of liver)
0	90±3	3.7±0.3	18.5±0.7
1	92±1	3.8±0.2	21.3±0.3
2	97±2	4.3±0.3	23.4±0.7
3	102±3	5.0±0.2	26.3±1.3
7	115±4	7.0±0.6	31.2±2.5

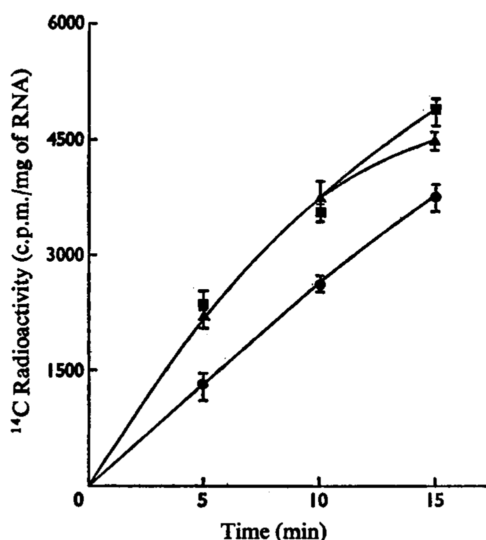

Fig. 1. *Amino acid incorporation by isolated liver microsomes of normal and hexachlorobenzene-treated rats*

[U-^{14}C]Leucine incorporation by isolated microsomes was studied in a cell-free system under conditions described by Munro *et al.* (1964). The other experimental details are given in the text. Each point represents the mean value obtained from three experiments. Vertical lines indicate the maximum and minimum values obtained for each point. ●, Control; ▲, 24h-treated rat; ■, 48h-treated rat.

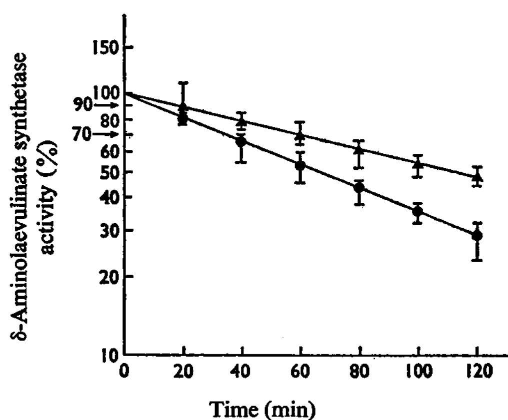

Fig. 2. *Degradation rate of liver δ-aminolaevulinate synthetase in normal and hexachlorobenzene-treated rats*

The experimental details are given in the text. Each point represents the mean value obtained from three experiments. Vertical lines indicate the maximum and minimum values obtained for each point. ●, Rats treated with allylisopropylacetamide; ▲, rats treated with hexachlorobenzene.

for haemoprotein synthesis as well as for breakdown, no experimental technique is available to detect it since almost the entire haem in liver remains bound to haemoproteins.

Manifestation of porphyria due to feeding with hexachlorobenzene is associated with increased activity of δ-aminolaevulinate synthetase (Tables 1 and 2). The greater accumulation of uroporphyrins can be due to a block in the utilization of uroporphyrins after prolonged feeding with the drug, as suggested by Taljaard *et al.* (1971). It may also be pointed out that under conditions in which amounts of cytochrome *P*-450 increase without a concomitant increase in δ-aminolaevulinate synthetase activity there is no over-production of the porphyrins or their precursors. The slow and moderate increase in δ-aminolaevulinate synthetase activity contrasts with the immediate and striking inductive effects on the

enzyme by drugs such as allylisopropylacetamide and dicarbethoxydihydrocollidine. The results presented in Fig. 2 indicate that the half-life of the enzyme is enhanced after prolonged feeding of the animals with hexachlorobenzene as compared with the results obtained with allyl isopropyl acetamide. Thus there is a possibility that prolonged feeding of hexachlorobenzene might lead to elevated activity of δ-aminolaevulinate synthetase by decreasing the degradation rate of the enzyme rather than by accelerating the rate of synthesis.

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