hypomagnesaemia observed in the calves examined by Herman (1936). The results of this worker remain irreconcilable with those of other workers in the field.

According to Bechdel, Hilston, Guerrant & Dutcher (1938) the minimum requirement of normally fed calves for vitamin D is about 6·7 i.u./kg. body wt./day. This figure was, however, derived from experiments in which a diet rather low in phosphorus was given (Ca:P about 3:1). More recently Thomas & Moore (1951), using a better diet in this respect (Ca:P about 1·3–1·8:1), have reported that calves grew normally for 8 months with no blood abnormalities and no signs of rickets when their vitamin D intake was only about 4·8 i.u./kg. body wt./day (less during the first 3 months). Post-mortem examination showed normal bone formation and normal values for bone ash in these animals. Our calves (1A, 2A, 4A, 7A, 8A) which were given 300–400 i.u. of supplementary vitamin D/day from 5 weeks of age received a total intake of vitamin D (or its equivalent as naturally occurring vitamin D activity in the milk) of between about 4·5 and 7·0 i.u./kg. body wt./day. They therefore received dietary vitamin D in amounts which appear to be adequate for normally fed calves. Our results show, however, that this vitamin D intake was not sufficient for optimum calcium utilization in our calves. This regime was not continued for a sufficiently long time to provide evidence on whether any ill effects were potentially associated with the impaired calcium utilization, but hypocalcaemia has been shown in milk-fed calves on even higher vitamin D intakes (Smith, 1957; Parr, 1957). The available evidence favours the view that vitamin D requirements are greater in milk-fed calves than in normally fed calves.

Irradiation of some calves with u.v. light appeared to produce an effect on calcium metabolism similar to that produced by giving vitamin D in large amounts (70 000 i.u./day).

SUMMARY

1. The addition of a supplement of 300–400 i.u. of vitamin D_3/day to the diet of milk-fed calves reduced the rate of decrease in calcium utilization as the calves got older and prevented the plasma-calcium level from falling when the plasma-magnesium level fell.

2. The addition to the diet of 70 000 i.u. of vitamin D_3/day or irradiation of the calves with u.v. light greatly improved the utilization of calcium both in calves previously receiving no vitamin D and in those receiving 300–400 i.u. of vitamin D_3/day.

3. The mean faecal excretion of magnesium increased from 32% of the dietary magnesium at about 3 weeks of age to 86% at about 16 weeks of age, after which it did not change greatly. Neither the addition of vitamin D to the diet nor irradiation of the calves with u.v. light had any appreciable effect on this excretion.

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The Inhibition of Mitosis by the Reaction of Maleic Hydrazide with Sulphydryl Groups

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Maleic hydrazide, or 6-hydroxy-2*H*-pyridazin-3-one, inhibits the growth of plants (Leopold & Klein, 1951) and has been used to control the sprouting of stored potatoes and onions. McLeish (1953) demonstrated that the site of action of the chemical was on the chromosomes of the *Vicia* cell, so affecting the early stages of mitotic division. Studies on the effect of maleic hydrazide on the enzymes of intact cells suggest that maleic hydrazide affects the enzymes requiring sulphydryl (SH) groups for activity (Andrae & Andrae, 1953; Bertossi, 1955; Muir & Hansch, 1953; Isenberg, Odland, Prop & Jensen, 1951). No reaction, however, between maleic hydrazide and soluble SH

groups has been demonstrated *in vitro* at physiological pH values (Leopold & Price, 1956) or in the presence of tissue extracts (Weller, Ball & Sell, 1957). These findings differ from the known reactions between other maleic acid derivatives and the SH group present in glutathione (GSH) and proteins. These reactions result in the addition of the SH group to the double bond of the maleic acid (Morgan & Friedman, 1938).

The present study is an attempt to bring some consistency to these contrasting results by comparing the reactions of maleic hydrazide and SH compounds *in vivo* and *in vitro* and also to obtain information on the biochemical mechanism of mitosis. The germinating pea seed was chosen as experimental plant material since S. P. Spragg & E. W. Yemm (unpublished work) have shown that considerable changes in glutathione occur when this seed germinates. Analyses for the GSH in the intact seeds and in the separated axes (plumules and radicles) were combined with a study of inhibition of enzymes by maleic hydrazide. Inhibition of division was found to be associated with interference with SH metabolism.

MATERIALS AND METHODS

Treatment of the pea seeds. Pea seeds of the variety Meteor were used in all the experiments. The procedure used for treating the seeds was to soak these for 48 hr. at 20° and then to transfer them to moist soil. The time of first wetting the seeds was taken as zero time for the experiments. Under these conditions between 90 and 100% of seeds soaked in water germinated normally.

Maleic hydrazide solution. Pure maleic hydrazide was dissolved in the appropriate buffer solutions for the enzyme reactions, and in water for treating the seeds. These solutions were found to be stable for at least 1 month at room temperatures.

p-*Chloromercuribenzoate.* An approx. 4 mM-solution was obtained by dissolving the acid in water and adjusting the pH with N-NaOH to the value used in the experiment.

Glutathione. A commercial preparation was used; iodate titrations showed that the sample contained at least 90% of GSH. *N*-Ethylmaleimide, glucose 1-phosphate (dipotassium salt), hexose diphosphate (disodium salt) and casein were commercial preparations.

Determination of colour intensity. In all the determinations the intensity of the colours was measured with the EEL portable colorimeter (Evans Electroselenium Co. Ltd., Harlow, Essex).

Buffer solutions. The acid buffer used was 0·2 M-sodium acetate buffer adjusted to pH 5·4; the neutral buffer was a 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) adjusted to pH 7·4. The pH of the enzyme digests did not alter during the reaction.

Estimation of glutathione and oxidized glutathione. GSH and oxidized glutathione (GSSG) were extracted by grinding the seeds with cold 2·5% (w/v) sulphosalicylic acid, following the method described by S. P. Spragg & E. W. Yemm (unpublished work). The nitroprusside method of Grunert & Phillips (1951) was used to estimate the GSH, by measuring the intensity of the red colour (Ilford filter no. 624). The method is not specific for GSH since other SH compounds will react with the nitroprusside. However, Hopkins & Morgan (1943) isolated GSH from freshly germinated pea seeds and S. P. Spragg & E. W. Yemm (unpublished work) showed that the predominating soluble SH compounds in germinating Meteor pea seeds were GSH and GSSG. In view of these results the soluble SH compounds of the cotyledons and the axes have been called GSH and GSSG. Total glutathione was determined by repeating the above estimation on a second sample of the extract after electrolytic reduction of the GSSG by the method of Dohan & Woodward (1939). The values for GSH and GSSG did not vary more than ±10% from the mean of duplicate extractions.

Estimation of β-amylase activity. A commercial preparation of β-amylase was incubated with soluble starch under the conditions given in the legend to Fig. 1. The reducing sugar produced by the hydrolysis of the starch was estimated by the Somogyi copper method (Somogyi, 1945). This method gave results repeatable to ±5% of the mean of duplicate samples each containing 100 μg. of glucose.

Preparation and estimation of the activity of potato-starch phosphorylase. A partially purified solution of potato-starch phosphorylase was prepared and its activity was determined by the procedures described by Hanes (1940). The enzymic conversion of glucose 1-phosphate into starch was carried out in 10 ml. centrifuge tubes, and after incubation of the mixtures (conditions and concentrations are given in the legend to Fig. 2) the enzyme was inactivated by the addition to the tubes of an equal volume of 10% (w/v) trichloroacetic acid. The precipitates were removed by centrifuging and the free inorganic phosphate in the supernatant solutions was estimated by the method of Peel & Loughman (1957). The blue colour produced was measured by means of the Ilford red filter no. 608. Estimations of phosphatase activity were obtained by determining the inorganic phosphate liberated from the glucose 1-phosphate in the absence of soluble starch. In all the preparations of the enzyme used, the phosphatase activity was negligible compared with that of the phosphorylase enzyme. The method yielded results repeatable to within ±10% of the mean of duplicate determinations of activity.

Preparation and estimation of activity of aldolase. A partially purified preparation of aldolase was isolated from germinating pea seeds by ammonium sulphate precipitations by means of the initial steps in the procedure described by Stumpf (1948). The activity was determined by the method of Beck (1955). The resultant colour intensity was measured with the yellow-green Ilford filter no. 625. Enzyme blanks were prepared by adding the hexose diphosphate to the solution after precipitation of the enzyme.

Estimation of the activity of trypsin. A volume (0·3 ml.) of 0·01% solution of crystalline trypsin (L. Light and Co., Colnbrook, Bucks) was treated at 32° with 3 ml. of casein solution (1% in 0·1 M-tris, pH 7·0) and 4 ml. of 10 mM-MH in 0·1 M-tris, pH 7·0, or water. Samples of the reaction mixture were removed at intervals and the enzyme was inactivated by the addition of an equal volume of 10% trichloroacetic acid. After centrifuging, the free amino acids in the supernatant were determined by the method of Yemm & Cocking (1955). The intensity of the blue colour was measured with the aid of the Ilford yellow-green filter

no. 625. This method gave results repeatable to within ±5% of the mean duplicate estimations.

Estimation of protein SH *groups.* The SH groups of β-amylase were estimated by titration with 0·01 N-$AgNO_3$, by the amperometric method of Benesch, Lardy & Benesch (1955).

RESULTS

Inhibition of growth of pea seedlings

During the early stages of germination of the pea seed maleic hydrazide had no visible effects on the emergence of the radicle from the enclosing testa. As the radicle enlarged, the inhibitory effects of mM-maleic hydrazide became apparent, and after 4–5 days the treated seedlings stopped growing. This inhibition of growth appeared to be permanent since no further growth occurred when the seedlings were left for a further 20 days in the soil. At the 4- to 5-day stage, the tip of the treated radicle appeared rounded and slightly bulbous in shape, the hypocotyl was swollen and, if the plumule did emerge, the small leaflets were pointed and did not increase in size. These treated seedlings differed markedly from the control seedlings where normal growth was associated with a long slender radicle tapering to a point. Microscopical examination of the cells of the growing point of treated seedlings showed that very few of the cells were dividing. Furthermore, the few cells which were dividing had mitotic figures characteristic of the early stages of mitotic division. This state contrasted sharply with that in the controls where cells were present in all stages of division. Examination of developing pea seedlings showed that the initial growth of the radicle was mainly by cell enlargement, and maleic hydrazide had no visible effects on this process.

Table 1 shows the results of one experiment illustrating the effect of maleic hydrazide on the amounts of GSH and GSSG in the intact seeds during the first 42 hr. of germination. Germination was accompanied by an increase in the amount of GSH in both the treated and control seeds and a decrease in the quantity of GSSG. Relatively small quantitative differences were found between the two sets of seeds in that more GSH was detected in the seeds treated with maleic hydrazide.

In one experiment the seeds were treated with different concentrations of maleic hydrazide and estimates were obtained of the amounts of GSH and GSSG in the axes after 4 and 10 days of growth, and the results are shown in Table 2. After 4 days, the quantity of GSSG/unit fresh weight ranged from 160 μg. in the water control to zero in mM-maleic hydrazide. With one exception, the decrease in GSSG was relatively uniform as the maleic hydrazide concentrations were increased.

Table 1. *Effects of* 10 mM-*maleic hydrazide on the reduced and oxidized glutathione in germinating pea seeds*

Time (hr.)	Treated (μg./seed)		Control (μg./seed)		100 × Treated/control	
	GSH	GSSG	GSH	GSSG	GSH	GSSG
16	130	78	154	60	84·4	130
42	220	52	191	47	115	110

Table 2. *Effects of different concentrations of maleic hydrazide on the content of reduced and oxidized glutathione and the germination of pea seedlings*

Seeds were soaked for 48 hr. and then planted. Analyses were made on the plumule plus radicle only.

Concn. (mM) ...	1·0	0·66	0·33	0·1	Control
		(μg./g. fresh weight)			
		After 4 days			
GSH	567	510	640	410	476
GSSG	0·0	83	209	120	166
Total	567	593	849	530	642
Total $\frac{GSH}{GSSG}$	∞	6·17	3·2	3·4	2·87
		After 10 days			
GSH	256	211	182	195	164
GSSG	73	78	123	133	100
Total	329	289	305	328	264
Total $\frac{GSH}{GSSG}$	3·5	2·7	1·48	1·48	1·64
% Germination	25·0*	30·0*	48·0*	89·0	86·0

* Seedlings did not appear normal at 10 days and did not grow above 5 cm. high.

In contrast, the amount of GSH increased as the concentration of maleic hydrazide increased. The high figures for GSH and GSSG in the seedlings treated with 0·33 mM-maleic hydrazide were caused by an abnormally low fresh weight in this sample. The relationship between GSH and GSSG at varying concentrations of maleic hydrazide was emphasized by expressing the results as the ratio GSH:GSSG. This ratio increased steadily as the concentration of maleic hydrazide increased. The absence of GSSG in the samples treated with maleic hydrazide at the 4-day stage of growth was confirmed by several experiments. In general, the amount of GSH was higher in the treated seedlings than in the controls.

A further sample of seedlings was analysed after 10 days, and a similar though less marked effect of maleic hydrazide on the ratio GSH:GSSG was found. Slightly more total glutathione was detected in the treated seedlings than in the control. This effect was probably the result of comparing seedlings growing normally with those whose growth had been arrested, for it has been found that a decrease in the total GSH found is associated with normal growth. The percentage germination was assessed and it was most noticeable that this increased with decreasing concentration of maleic hydrazide and, hence, with increasing amounts of GSSG, although normal growth of the seedling after this stage was found only in the 0·1 mM-solution.

Summarizing, the major effect of maleic hydrazide on the glutathione of the dividing cells of the axis was to disturb the equilibrium between GSH and GSSG, so leading to an accumulation of GSH in the tissues. Comparison of these effects with the results found for seeds in the early stage of germination, where the equilibrium was unaffected, shows that glutathione functions in at least two differing systems, one of which is important to mitotic division.

Inhibition of enzymes with maleic hydrazide

The results of the analyses on growing tissue show that maleic hydrazide interferes in SH metabolism, although no direct reaction has been demonstrated between the soluble GSH compounds and maleic hydrazide *in vitro* (Weller *et al.* 1957). The following experiments show that enzymes requiring SH groups for activity are inhibited by maleic hydrazide in an irreversible manner.

The effect of maleic hydrazide on β-amylase, an enzyme requiring free SH groups for activity (Weill & Caldwell, 1945), is shown in Fig. 1. The progress curves for the action of the enzyme on soluble starch show that in the presence of maleic hydrazide the amount of reducing sugar liberated in 15 min. at pH 5·0 was approx. 53% of the control. Similarly, at pH 7·3 the amount of sugar liberated was 61% of the control in the same time (this figure was obtained from an experiment separate from that quoted in Fig. 1). These inhibitory effects were confirmed in other experiments, where it was found that the inhibition increased if the enzyme solution was stored for a day or more at 5°. These storage conditions did not materially affect the rates of sugar production for the controls and the explanation of this change is not known.

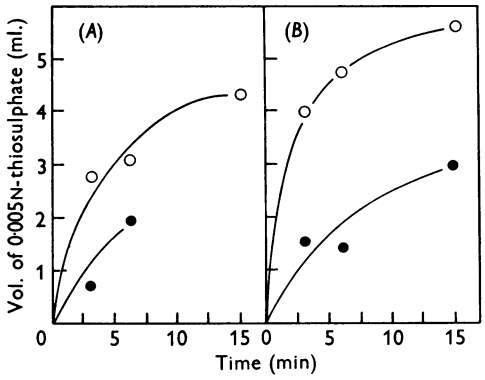


Fig. 1. Progress curves of β-amylase in the presence of maleic hydrazide (●) and water control (○). pH values of the digest were 7·8 (*A*) and 5·0 (*B*). A volume (0·5 ml.) of enzyme (1 μg./ml.) was incubated at 32° with 2 ml. of buffer [(*A*) 0·1 M-tris; (*B*) 0·2 M-acetate] and either 12·5 ml. of water or 6·5 ml. of water plus 6 ml. of 10 mM-maleic hydrazide.

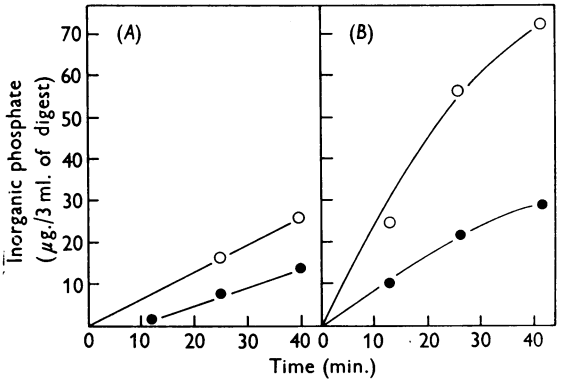


Fig. 2. Progress curves of starch phosphorylase in the presence of maleic hydrazide (●) and water (○). pH values of the digests were 7·4 (*A*) and 5·2 (*B*). A volume (0·5 ml.) of enzyme (11·5 mg. of N/ml.) was incubated at 32° with 0·1 ml. of soluble starch (1%), 0·3 ml. of buffer [(*A*), 0·1 M-tris; (*B*) 0·2 M-acetate] and 1 ml. of either 10 mM-maleic hydrazide or water. The reaction was started by the addition of 0·1 ml. of glucose 1-phosphate (46 mg./ml., dipotassium salt).

Further evidence for maleic hydrazide inhibition through inactivation of SH groups on the protein chain was obtained with starch phosphorylase, an enzyme which also requires the presence of free SH groups for activity (Elliot, 1946). The progress curves for the liberation of phosphate from glucose 1-phosphate as a result of starch synthesis are shown in Fig. 2. As with β-amylase, the activity of the enzyme was reduced by maleic hydrazide at the two pH values studies. After 48 min. the phosphate liberated in the presence of maleic hydrazide was 54 and 41 % of the controls at pH 7·4 and 5·8 respectively. For comparative purposes the activities of the same enzyme preparation were inhibited with *p*-chloromercuribenzoate and *N*-ethylmaleimide and the results are shown in Table 3. Both *p*-chloromercuribenzoate and maleic hydrazide were more active inhibitors than *N*-ethylmaleimide, but at equal molar concentrations maleic hydrazide was not so effective an inhibitor as *p*-chloromercuribenzoate for this enzyme. The phosphatase activity of the enzyme preparations remained low and unaffected by the maleic hydrazide.

Sulphydryl enzymes can be protected against the action of *p*-chloromercuribenzoate or iodoacetamide by GSH or cysteine. Attempts were made therefore to decrease the maleic hydrazide inhibition of starch phosphorylase by the inclusion of GSH in the reaction mixture. The concentration of GSH used was sufficient to decrease the effective concentration of maleic hydrazide by half if any reaction occurred between the two chemicals. Tests had shown that if the maleic hydrazide was decreased to this amount its inhibition of this enzyme preparation was small. The results given in Table 4 (with a different enzyme preparation from the previous experiments) show that GSH does not affect the maleic hydrazide inhibition at either pH value used. These results are in agreement with the previous data which show that maleic hydrazide inhibition of growth occurs even in the presence of excess of GSH *in vivo* (Table 2).

The effect of maleic hydrazide on the activity of two further enzymes was studied. The two examined, trypsin and pea aldolase, contrasted with the previous enzyme in that they do not require free SH groups on the protein for activity. The activity of trypsin, however, is dependent on the presence of S·S groups on the protein (Liener, 1957). Previous investigators have demonstrated the presence of SH groups on purified aldolase proteins (Benesch *et al.* 1955), but since *p*-chloromercuribenzoate did not inhibit the crude enzyme preparation these are not essential for activity. Maleic hydrazide did not inhibit the activity of aldolase or trypsin under the conditions used.

Table 3. *Inhibition of potato-starch phosphorylase by N-ethylmaleimide, maleic hydrazide and p-chloromercuribenzoate*

A volume (0·5 ml.) of enzyme (11·5 mg. of N/ml.) was incubated at 32° with 0·1 ml. of 1 % soluble starch, 0·3 ml. of 0·1 M-tris buffer, pH 7·4, 0·1 ml. of glucose 1-phosphate (46 mg./ml., dipotassium salt) and 1 ml. of either 10 mM-maleic hydrazide or 10 mM-*N*-ethylmaleimide, or 5 mM-*p*-chloromercuribenzoate or water. Results have been corrected for blanks.

	μg. of P/3 ml./15 min.	Inhibited (%)
Control	18·9	—
N-Ethylmaleimide	13·9	73·5
Maleic hydrazide	10·0	53·5
p-Chloromercuribenzoate	9·7	51·0

Table 4. *Inhibition of potato-starch phosphorylase by maleic hydrazide in the presence of glutathione*

A volume (0·5 ml.) of enzyme was incubated at 32° with 0·1 ml. of 1 % soluble starch, 0·3 ml. of 0·1 M-tris buffer or 0·2 M-acetate buffer, 0·1 ml. of glucose 1-phosphate (46 mg./ml., dipotassium salt), 0·5 ml. of GSH (3·3 mg./ml.) and 1 ml. of 10 mM-maleic hydrazide or water. Results have been corrected for blanks.

	μg. of P/3 ml./15 min.	
	pH 7·4	pH 5·4
Maleic hydrazide + GSH	8·0	11·6
Control	14·7	23·7
$100\left(\frac{\text{Maleic hydrazide + GSH}}{\text{Control}}\right)$	54·3	48·2

These experiments suggest that maleic hydrazide inhibits enzymes requiring free SH groups for activity, but more detailed experiments are necessary to explain the mechanism of this inhibition. The inability of maleic hydrazide to inhibit trypsin shows that maleic hydrazide cannot destroy the S·S grouping present on the protein chain.

Effect of maleic hydrazide on the SH groups of amylase

Further evidence that protein SH groups and maleic hydrazide combine was obtained by titrating the SH groups remaining on β-amylase protein after treatment with maleic hydrazide. Two digests, one with and one without maleic hydrazide, were incubated under the experimental conditions of Fig. 1 and, after 90 min., 2 ml. samples were taken. The enzyme protein was denatured with 8 M-urea, following the conditions given by Benesch *et al.* (1955), and the free SH groups were titrated with 0·01 N-$AgNO_3$, by an amperometric method. Treatment with urea was necessary since the control sample showed no uptake of Ag^+ ions before denaturation. However, the treated sample, which gave the same titre before and after denaturation, was found to contain only 40 % of the free

SH groups found in the control sample (typical figures being 0·43 μM-SH/2 ml. for the control sample compared with 0·18 μM-SH/2 ml. for the maleic hydrazide-treated sample).

Maleic hydrazide-treated and untreated β-amylase solutions were incubated for 90 min. at 27° with GSH (concentration of GSH was 2·5 mM) and 2 ml. samples titrated. To prevent the interference of GSH in the titration of the protein SH groups, 75% of the GSH was removed by the addition of *N*-ethylmaleimide to both the treated and untreated samples. After denaturation with urea the untreated protein SH was 0·55 μM-SH/2 ml., compared with 0·2 μM-SH/2 ml. for the treated protein. However, after dialysis of both the treated and untreated protein solutions against distilled water for 18 hr. at 5°, the protein SH groups were the same. Thus dialysis appears to remove maleic hydrazide from the SH groups on the protein chain. The significance of this result is not clear but it seems possible that the maleic hydrazide is removed by a mass-action effect.

Similar experiments with starch phosphorylase (Table 4) suggested that the inhibition by maleic hydrazide was not reversed by GSH and more direct evidence for this was found by amperometric titration.

Ultraviolet absorption of maleic hydrazide and maleic anhydride

Qualitative tests with bromine water and permanganate solutions showed that at least one of the double bonds in the molecule is ethylenic in character and thus similar to the double bond in maleic anhydride.

The u.v. light-absorption spectra of maleic hydrazide shows that the molecule is stable in the enolic form (6-hydroxy-2*H*-pyridazin-3-one) (Miller & White, 1956). These results have been confirmed and show that in 0·1 N-HCl, maleic hydrazide has two absorption maxima, one at less than 210 mμ and the second at 300 mμ (Fig. 3). These maxima shifted in 0·07 N-NaOH to 219 and 330 mμ respectively. In contrast, maleic anhydride has only one maximum below 210 mμ in 0·1 N-HCl, and at 220 mμ in 0·07 N-NaOH, so corresponding with one of the maxima in the maleic hydrazide absorption curve. In equal molar concentrations maleic hydrazide absorbed more strongly than the anhydride at 210 mμ in acid solution.

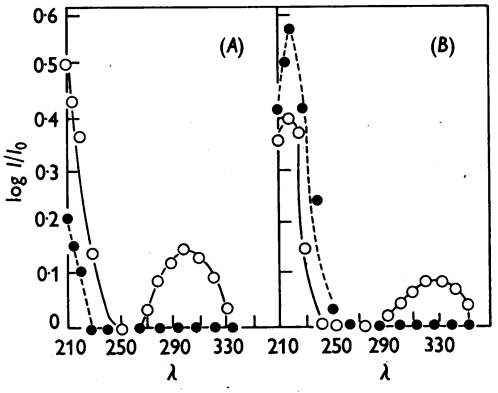


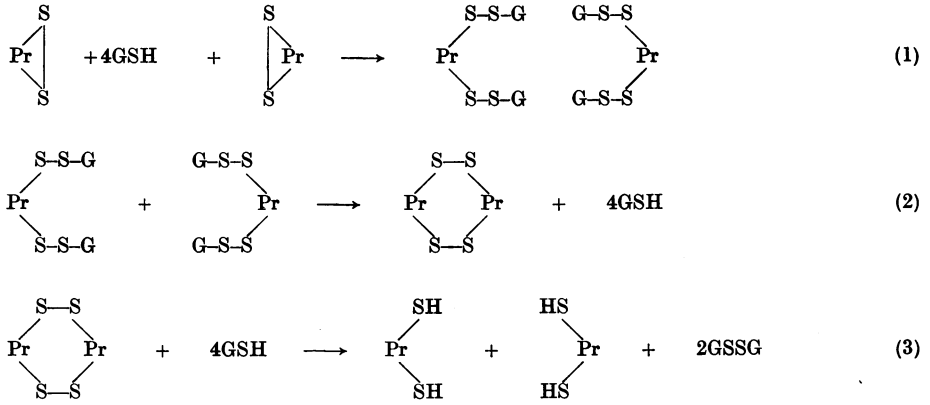
Fig. 3. U.v. light-absorption curves of maleic hydrazide (○) and maleic anhydride (●). (*A*), Solvent 0·1 N-HCl, $\lambda_{max.}$ for maleic hydrazide at 300 mμ; (*B*), solvent 0·07 N-NaOH, $\lambda_{max.}$ for maleic hydrazide at 218 mμ and 320 mμ; $\lambda_{max.}$ for maleic anhydride at 220 mμ.

The presence of one maximum common to both the anhydride and maleic hydrazide, together with the marked reactivity of maleic hydrazide to the double-bond reagents, suggest that the double bond between the carbon atoms of maleic hydrazide possesses a certain proportion of the high reactivity characteristic of maleic acid derivatives. This reactivity is not sufficient to allow maleic hydrazide to condense with SH groups of glutathione and other soluble thiol compounds in the manner of other maleic acid derivatives.

DISCUSSION

S. P. Spragg & E. W. Yemm (unpublished work) have shown that in the early stages of germination of pea seeds there occurs a conversion of GSSG into GSH. This change in the redox equilibrium of glutathione was not affected by maleic hydrazide, showing that in intact tissue maleic hydrazide and GSH do not interact. It is also inferred from these results that maleic hydrazide has negligible effect on the functioning of the enzyme systems associated with the oxidation and reduction of glutathione (Mapson & Goddard, 1951; Mapson & Moustafa, 1956).

The growth of the radicle in the germinating seeds can be divided into two stages. The first is the emergence of the radicle through the testa and is mainly the result of cell enlargement of pre-existing cells; the second follows with mitotic division contributing to a major proportion of the growth. Maleic hydrazide did not affect the cell enlargement, but influenced the equilibrium between GSSG and GSH during division. Furthermore, the ability of high concentrations of maleic hydrazide to effect a complete conversion of GSSG into GSH in the dividing cells suggests that during mitosis in the treated tissue either there is an increase in the activity of the enzyme systems reducing glutathione relative to the activity of those oxidizing it or that other reactions which specifically require glutathione are inhibited. As the axis aged there was a partial recovery of the equilibrium between the two redox forms.



These results indicate that, in addition to its many known biochemical reactions, glutathione participates in reactions specific to cells preparing to divide. In view of the reaction between maleic hydrazide and the SH groups on proteins it appears that the additional role involves a reaction between glutathione and protein SH groups, the maleic hydrazide intervening and preventing normal functioning of this reaction. The effect of varying the concentration of maleic hydrazide (Table 2) is of interest in relation to the importance of the glutathione–protein SH cycle, since the continued growth of the axis was found to be associated with the maintenance of the GSH:GSSG ratio.

Mazia (1954) has summarized the evidence of previous investigators, which shows that GSH is intimately involved in the preliminary stages of mitotic division of sea-urchin eggs, and he has suggested that the reactions (1) and (2) above (in which Pr represents protein) occur during the division of the fertilized egg.

This sequence of reactions points to the importance of glutathione and protein SH groups in mitotic division in animal cells. The present results show that important reactions also occur between SH groups in the dividing plant cell. The fact that GSH accumulates at the expense of the GSSG in the maleic hydrazide-treated cells (Table 2) suggests that the maleic hydrazide has blocked a process which in the normal cell reoxidizes GSH. In view of this evidence it seems possible that reaction (3) may represent the process of oxidation.

In a comparative study of a non-dividing mutant of *Candida albicans*, Nickerson & Falcone (1956) found that division of the fungal cell was associated with the presence of an enzyme which reduces protein disulphide groups. It seems possible that reaction 3 may be catalysed by a similar type of enzyme. The dynamic state of this process is dependent on the continual re-formation of GSH from GSSG. This conversion may take place by reduction with reduced triphosphopyridine nucleotide (Mapson & Goddard, 1951), although no definite evidence is available to show that this compound is the only one capable of reducing GSSG in the dividing cell.

The structural similarities which were found to exist between maleic hydrazide and maleic anhydride suggest that the maleic hydrazide may form an addition compound with some SH groups in the protein molecule, possibly before the oxidation step, and this prevents the subsequent breaking of any disulphide groups which may have formed during the preliminary steps of division. In fact, the experiments with maleic hydrazide suggest that, in plant cells preparing to divide, the maintenance of certain protein SH groups is essential for subsequent normal mitotic division.

Finally, McLeish (1953) has shown that maleic hydrazide can produce chromosome aberrations, and it is of interest to speculate on the mechanisms involved in this process and their relationships with SH groups during mitosis. A reaction could take place between two SH groups and one molecule of maleic hydrazide in such a manner that two protein chains become linked irreversibly. In the subsequent migration of the proteins to form the two new cells, the chains joined by maleic hydrazide must either move as a unit, or split so that each new molecule consists of proteins of the old molecule condensed via the maleic hydrazide. In either case the genetic constitution may be affected by an unbalanced movement of the proteins and the formation of the unnatural links between the peptide chains. On this basis part of the normal course of division would be the controlled formation of the S·S bonds between specific proteins followed by reduction of these bonds by GSH.

SUMMARY

1. Pea seeds treated with maleic hydrazide germinated normally until cell enlargement was replaced by cell division, when growth stopped.

2. The ratio of reduced (GSH) to oxidized (GSSG) glutathione, i.e. GSH:GSSG, in cotyledons was not affected by maleic hydrazide but at the onset of cell division GSH increased at the expense of GSSG in treated axes only.

3. Certain enzymes requiring free sulphydryl groups (starch phosphorylase and β-amylase) were inhibited irreversibly by maleic hydrazide, but other enzymes (trypsin and pea aldolase) were not affected.

4. The results suggest that maleic hydrazide reacts with protein SH groups and thus inhibits the reduction of protein S·S by GSH during mitosis.

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Formation of Antibody by Isolated Perfused Lungs of Immunized Rabbits

THE USE OF [^{14}C]AMINO ACIDS TO STUDY THE DYNAMICS OF ANTIBODY SECRETION

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Under the usual experimental conditions yields of newly formed proteins secreted by tissue slices are too small to permit their isolation for study without carrier protein, and when whole organs, such as the liver, are perfused with blood (e.g. Miller, Bly, Watson & Bale, 1951) recovery of any newly synthesized plasma proteins is complicated by admixture with similar proteins already present in the perfusion fluid.

Normal lung forms little or no γ-globulin, nor is it known to secrete any other protein. When rabbits are hyperimmunized by intravenous injections of a particulate antigen, such as pneumococci type 3, the lung may become a major site for antibody formation, as shown both *in vivo* (Humphrey & Sulitzeanu, 1958) and by tissue slices *in vitro* (Askonas & Humphrey, 1958). As a result of hyperimmunization there is enormous proliferation of lymphoid tissue and plasma cells around the small arteries and the lung may increase two to four times in weight. If such lungs are perfused with salt medium containing normal