though a considerable part of it may exist as readily hydrolysable bound glutamic acid of as-yet undetermined structure.

4. The amounts of pyrrolidonecarboxylic and γ -aminobutyric acid found in extracts of fresh grass are very small, and pyrrolidonecarboxylic acid probably does not exist as such in the grass, the little found being formed during the extraction.

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Studies on Biosynthesis of Cholesterol

8. THE EFFECT OF Mg^{2+} AND Mn^{2+} IONS AND OF ADENOSINE TRIPHOSPHATE ON ENZYMIC ACTIVITY OF MICROSOMES IN STEROL BIOSYNTHESIS FROM MEVALONIC ACID*

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It was shown in previous studies that for the synthesis of squalene and cholesterol from mevalonic acid by cell-free preparations of rat liver a fraction of soluble protein and microsomes were needed and that adenosine triphosphate and reduced pyridine nucleotides were essential cofactors in this enzyme system (Popják, Gosselin, Gore & Gould, 1958). It was further demonstrated that during aerobic conditions, when mostly cholesterol was synthesized, a reducing agent, such as glutathione or ascorbic acid, was needed to activate the system.

The soluble enzymes could be preserved very satisfactorily, after precipitation with ammonium sulphate at -15° , but the microsomes progressively lost their activity when kept frozen at -15° or at -79° . Consequently in our previous work microsomes were prepared freshly for each experiment.

* Part 7: Cornforth, Cornforth, Pelter, Horning & Popják (1959).

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The present paper describes a study of the 'ageing' process of the microsomes and of the conditions leading to the restoration of the ability of old preparations of microsomes to catalyse the synthesis of sterol from mevalonic acid in the presence of dialysed soluble enzymes and of the appropriate coenzymes. It was possible to reactivate fully preparations of microsomes that were kept frozen at -15° for 4 months and which had lost, apparently, 90% of their activity. A brief account of the results has already been presented (Gosselin & Popják, 1958).

METHODS

The methods and materials used in the present investigation were essentially those previously described (Popják *et al.* 1958).

Testing of enzymic activity of microsomes. The criterion of enzymic activity of the microsomes was their ability to catalyse the synthesis of digitonin-precipitable sterols from [$2-^{14}C$]mevalonate when combined with dialysed soluble liver enzymes in the presence of adenosine triphosphate

(ATP), pyridine nucleotides, nicotinamide, Mg^{2+} , ascorbate and oxygen. Since for the synthesis of sterols soluble enzymes as well as the cell particles are needed, the activity of the aged preparations of microsomes was usually assayed against an enzyme system which contained microsomes prepared on the day of the experiment or a few days previously, the same preparation of the soluble enzymes being employed in the control and test systems.

The dialysed soluble enzymes and microsomes were prepared as described previously (Popják *et al.* 1958) from homogenates made according to Bucher & McGarrah (1956).

When a preparation of microsomes is described as being 4 days old or 4 months old, it means that the suspension of microsomes (in 0.1M- KH_2PO_4 - K_2HPO_4 buffer, pH 7.5, containing 0.03M-nicotinamide and 4 mM- $MgCl_2$) was kept frozen at -15° for that length of time after the day of its preparation.

The suspensions of microsomes were so adjusted that 0.1 ml. contained an amount of microsomes equal to that present in 1 ml. of the liver homogenate from which they had been prepared.

Incubations. All incubations were made in conical flasks at 37° for 1 hr. with air as gas phase in the flasks. In most experiments a basic 'standard incubation mixture' was employed consisting of: dialysed soluble enzymes (1 ml., 30–50 mg. of protein); suspension of microsomes (0.2 ml.); reduced diphosphopyridine nucleotide (DPNH; 1 μ mole); triphosphopyridine nucleotide (TPN; 1 μ mole); ATP (10 μ moles); [2- ^{14}C]mevalonate (1 μ mole); potassium ascorbate (30 μ moles); KH_2PO_4 - K_2HPO_4 buffer, 0.1M, pH 7.5; nicotinamide, 0.03M; $MgCl_2$, 4 mM; final volume, 4 ml. Additions to, and deviations from, this standard mixture are indicated in the tables. The concentrations of the reagents in the 'standard incubation mixture' were found previously to be optimum for a liver-enzyme system in which freshly prepared microsomes were employed. Extraction of cholesterol, preparation of digitonides and assay of ^{14}C were all done as described by Popják *et al.* (1958). Since the yields of sterol digitonides were nearly identical in all incubations (about 7 mg.) and since the 'infinite thickness' count is proportional to the specific activity (1600 counts/min./2 cm.² at infinite thickness is equivalent to 10^{-3} μ C/mg. under our counting conditions), all results are expressed in terms of the infinite-thickness counts.

Artificial ageing of microsomes. This was done by incubating in a test tube the usual concentrated suspension of microsomes (prepared on the day of the experiments) at 37° for a period ranging from 15 to 60 min. At the end of the required period the preparations were cooled in ice and kept at ice temperature until the test incubations were set up.

DL-[2- ^{14}C]Mevalonic acid. This substance synthesized according to Cornforth, Cornforth, Popják & Gore (1958), was obtained from the Radiochemical Centre, Amersham, Bucks. Material with a specific activity of 0.2 μ C/ μ mole was used.

RESULTS

When microsomes stored for 1 month at -15° , or at -79° , were tested in combination with freshly prepared and undialysed soluble enzymes [supernatant of homogenates centrifuged at 105 000 g for

1 hr. (S_{105})], the particles appeared to have lost approximately one-half of their activity. On the other hand, when these same particles were tried in a reconstituted system, containing dialysed soluble enzymes and the recognized cofactors (Popják *et al.* 1958), they were unable to catalyse efficiently the synthesis of cholesterol from mevalonic acid. This observation suggested at first that the fresh S_{105} preparations and microsomes contained some common, as yet unidentified, factor, which was lost from microsomes during storage and was removed from the soluble enzymes by precipitation with ammonium sulphate and by dialysis.

Activation of microsomes stored at -15° by Mn^{2+} and Mg^{2+} ions and by adenosine triphosphate

Several unsuccessful attempts were made to preserve the activity of the stored microsomes by the addition of glutathione, cysteine or ascorbic acid to their suspensions before freezing or by the addition of coenzyme A and of vitamin B_{12} to the incubation mixtures. It was found, however, that preparations of microsomes, kept frozen at -15° for as long as 4 months, could be largely reactivated by Mn^{2+} or by an increased concentration of Mg^{2+} ions in the incubation mixture.

The results of the two experiments shown in Table 1 illustrate the strong 'rejuvenating' effect of mM- Mn^{2+} , in the presence of 4 mM- Mg^{2+} ions, on microsomes inactivated by storage at -15° for 7 and 11 weeks respectively.

Apart from manganese, increasing amounts of ATP and addition of sodium fluoride also had a reactivating effect on stored microsomes; this was further enhanced by the presence of Mn^{2+} (mM) (Table 2).

So far the results suggested that storage of microsomes at low temperatures caused both a loss of bivalent cations from the microsomes, leading to an inactivation of the enzymes, and possibly also a liberation of adenosine triphosphatase, which destroyed one of the essential coenzymes of the

Table 1. *Reactivation of stored microsomes by Mn^{2+} ions*

Each flask contained the standard incubation mixture (see Methods), but with 0.5 μ mole of TPN. Either freshly prepared or old microsomes were used as shown. The old microsomes employed in Expt. 1 were stored at -15° for 7 weeks and those in Expt. 2 for 11 weeks.

Microsomes used and additions	Specific activity of sterol digitonides (counts/min. at infinite thickness)	
	Expt. 1	Expt. 2
Fresh, none	1570	7438
Old, none	361	957
Old + mM- Mn^{2+}	1159	5004

Table 2. *Reactivation of aged microsomes by adenosine triphosphate, Mn²⁺ ions and by sodium fluoride*

Each flask contained the standard incubation mixture but with 15 μ moles of ATP and 2 μ moles of DPNH. Additions to this mixture were as shown.

Age of microsomes	Additions (μ moles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)
4 days	None	2177
	Mn ²⁺ , 4	4058
	ATP, 15	5157
	ATP, 15 + Mn ²⁺ , 4	6832
4 months	None	231
	Mn ²⁺ , 4	1259
	ATP, 15	2347
	ATP, 15 + Mn ²⁺ , 4	3369
	NaF, 40	606
	ATP, 15 + Mn ²⁺ , 4 + NaF, 40	5840

Table 3. *Cation requirements in sterol synthesis and ageing of microsomes*

Each flask contained: soluble enzyme, 1 ml.; suspension of microsomes stored previously at -15° , 0.2 ml.; DPNH, 2 μ moles; TPN, 1 μ mole; ATP, 30 μ moles; potassium ascorbate, 30 μ moles; [2-¹⁴C]mevalonate, 1 μ mole. The final volume (4 ml.) contained KCl (0.15 M) buffered to pH 7.5 with 2-amino-2-hydroxymethylpropane-1:3-diol/HCl (0.01 M) and nicotinamide (0.03 M). MgCl₂ and MnCl₂ were added as indicated. In Expt. A the microsomes used were 9 days and in Expt. B 11 weeks old.

Cation addition (μ moles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)	
	Expt. A	Expt. B
Mg ²⁺ , 16	3977	2551
Mg ²⁺ , 20	9810	3023
Mg ²⁺ , 16 + Mn ²⁺ , 4	9041	3620
Mn ²⁺ , 20	6710	2039

Table 4. *Cation requirement with fresh microsomes; comparison of Mn²⁺ with Mg²⁺ ions*

Each flask contained: soluble enzyme, 1 ml.; microsome suspension prepared on the same day in magnesium-free medium (0.1 M-potassium phosphate buffer, pH 7.5, + nicotinamide 0.03 M), 0.2 ml.; DPNH, 1 μ mole; TPN, 0.5 μ mole; ATP, 20 μ moles; ascorbate, 30 μ moles; [2-¹⁴C]-mevalonate, 1 μ mole. The final volume (4 ml.) contained 0.1 M-potassium phosphate buffer (pH 7.5) and nicotinamide (0.03 M). MgCl₂ and MnCl₂ were added as indicated.

Additions (μ moles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)
None	0
Mg ²⁺ , 16	8317
Mn ²⁺ , 16	4270
Mg ²⁺ , 16 + Mn ²⁺ , 4	6178
Mg ²⁺ , 20	7261
Mn ²⁺ , 20	4655
Mg ²⁺ , 8 + Mn ²⁺ , 8	8167

system. This view is supported by the fact that the highest activity resulted from incubating a mixture containing a high concentration of ATP, Mn²⁺ and of sodium fluoride.

In further experiments it was found that the effect of Mn²⁺ ions was not specific, as reactivation could be achieved with equivalent amounts of Mg²⁺ ions. This was shown by experiments presented in Table 3, in which a system partially reactivated by an increased concentration of ATP was used.

The need for the bivalent cations may be shown also in systems containing microsomes prepared on the day of the experiment: this need is absolute, as in the absence of either Mg²⁺ or Mn²⁺ ions the liver enzyme system fails to convert mevalonic acid into cholesterol (Table 4). However, in the presence of fresh microsomes a final concentration of 4 mM of Mg²⁺ or 2 mM-Mg²⁺ + 2 mM-Mn²⁺ appears to be the optimum, but Mn²⁺ cannot entirely replace Mg²⁺. The need for a higher concentration (5 mM) of bivalent cation became apparent only when aged preparations of microsomes were used, as was verified in several experiments; with fresh preparations of microsomes an increased concentration of Mg²⁺ (5 mM) or the addition of Mn²⁺ (mM) to 4 mM-Mg²⁺ ions did not enhance the synthetic activity of the enzyme system; on the contrary slight inhibiting effects were observed occasionally under such conditions.

Artificial ageing of microsomes

The inactivation of microsomes, which manifests itself in a need for Mn²⁺ in addition to Mg²⁺ ions, or for a greater concentration of Mg²⁺ and of ATP, proceeds slowly and variably at -15° , but it can

Fig. 1. Rapid ageing of microsomes at 37° . Each point is the result of an incubation carried out under standard conditions, except that the microsomes, prepared on the day of the experiment, were preincubated at 37° for 15–60 min. and then cooled in ice before use.

be much accelerated by incubation of microsomes at 37°. In the experiment recorded in Fig. 1 the microsomes used were prepared on the day of the experiment and their suspension (in 0.1M-phosphate buffer containing 0.03M-nicotinamide and 4 mM-MgCl₂) was incubated at 37° for 15–60 min. before use. If the ability of the microsomes, in the presence of soluble liver enzymes, to catalyse the synthesis of sterols from mevalonic acid in our standard incubation mixture is taken as a measure of their activity, it can be seen from Fig. 1 that the cell particles were inactivated rapidly and at a steady rate by previous incubation at 37°. The curve in Fig. 1 can be represented by a single exponential equation with a half-time of about 15 min. The inactivation of the microsomes at 37° is, apparently, equivalent to that induced by storage at low temperatures, as it can be fully reversed by increased concentrations of ATP and of Mg²⁺ ions [cf. Table 6, incubations (a)–(d)].

Relationship between concentrations of Mg²⁺ ions, adenosine triphosphate and of microsomes: appearance of inhibitors of sterol synthesis in aged preparations of microsomes

The main limiting factor in the observed activity of aged preparations of microsomes is the concentration of ATP used in the incubations. In previous studies (Popják *et al.* 1958) the optimum concentration of ATP for sterol synthesis from mevalonic acid was 2.5 mM (10 μmoles of ATP in 4 ml. incubation) when freshly prepared microsomes and 0.5–2.0 μmoles of DL-mevalonate were employed in the enzyme system; higher concentrations of ATP were often inhibitory. These results were confirmed in the present investigation, and it was found that there existed a close correlation between the amounts of ATP, Mg²⁺ and of microsomes in the incubations and the observed synthetic activity (Table 5). When fresh microsomes were used the

Table 5. *Magnesium, adenosine triphosphate and microsome interrelationship*

Each flask contained: soluble enzyme, 1 ml.; DPNH, 2 μmoles; TPN, 1 μmole; ascorbate, 30 μmoles; [2-¹⁴C]mevalonic acid, 1 μmole; the amounts of MgCl₂, ATP and of microsome suspensions used were as indicated. The final volume (4 ml.) contained 0.1 M-phosphate buffer (pH 7.5) and nicotinamide (0.03 M).

Microsome preparation	Vol. of microsome suspension (ml.)	Specific activity of sterol digitonides (counts/min. at infinite thickness)			
		Mg 16 μmoles ATP 10 μmoles	Mg 16 μmoles ATP 30 μmoles	Mg 20 μmoles ATP 10 μmoles	Mg 20 μmoles ATP 30 μmoles
Fresh*	0.4	5749	—	—	—
	0.2	5637	7453	4799	6089
	0.1	6039	2881	4968	5435
Aged†	0.4	613	—	—	—
	0.2	1569	4648	1968	5352
	0.1	3980	5147	2878	5006

* Microsomes prepared on the day of the experiment.

† Fresh microsomes preincubated at 37° for 30 min., then cooled in ice before use.

Table 6. *Reactivation of microsomes aged at 37° and the presence of inhibitors of sterol synthesis in aged preparations*

Each flask contained the standard incubation mixture except for the variations of the volume of microsome suspensions. The nature of the microsomes and additions was as shown. The reactivating factors [incubations (b), (d) and (h)] were: ATP, 20 μmoles; Mg²⁺, 4 μmoles and NaF, 40 μmoles. The fresh microsomes were prepared on the day of the experiment; the 'aged' microsomes used in incubations (c), (d) and (f) were fresh microsomes preincubated at 37° for 40 min. and then cooled down in ice before use. In incubations (g), (h) and (i) 'Sp' and 'Sd' represent the supernatant and sediment respectively of a suspension of microsomes kept at -15° for a few months and centrifuged at 105 000 *g* for 60 min. 'Sd' was resuspended in the usual amount of fresh buffer.

Nature of microsomes and additions	Specific activity of sterol digitonides (counts/min. at infinite thickness)
(a) Fresh microsomes (0.2 ml.)	4247
(b) Same as (a) + re-activating factors	7532
(c) Aged microsomes (0.2 ml.)	642
(d) Same as (c) + re-activating factors	7572
(e) Fresh microsomes (0.1 ml.)	2860
(f) Fresh and aged microsomes (0.1 ml. of each)	1228
(g) Fresh microsomes (0.2 ml.) + 'Sp' (0.2 ml.)	1958
(h) Same as (g) + re-activating factors	6041
(i) Fresh microsomes (0.1 ml.) + 'Sd' (0.1 ml.)	264

concentrations of these three components of the enzyme system were not very critical, except that a high concentration of ATP (30 μ moles/4 ml.) was inhibitory when the amount of microsomes was reduced to 0.1 ml. This inhibitory effect was largely counteracted by an increased concentration of Mg^{2+} ions (Table 5; experiment with fresh microsomes). After ageing of the particles by incubation at 37° this inhibitory effect of ATP disappeared and the high requirement for ATP became manifest.

The results of the experiment with the preincubated microsomes, shown in Table 5, also suggest that during ageing some inhibitory substance(s) might be released from the microsomes since, for a given level of ATP and Mg^{2+} concentration, an increase in the microsome content resulted in a proportionate lowering of the observed synthesis of sterol. It is shown in Table 6 that the addition of aged microsomes, or of a particle-free supernatant of old microsomes, to an enzyme system containing fresh microsomes resulted in a serious impairment of sterol synthesis. The inhibition caused by the supernatant of old microsomes was abolished by high concentrations of ATP, magnesium and sodium fluoride.

DISCUSSION

The main point which arises from the present observations is that ageing of the microsome suspensions (whether induced slowly at -15° or rapidly by incubation at 37°) causes an increased requirement for ATP and for Mg^{2+} (or Mn^{2+}) ions by the liver-enzyme system synthesizing sterol from mevalonic acid. Our observations on the reactivation of old microsomes by mM- Mn^{2+} ions in the presence of 4 mM- Mg^{2+} ions suggested at first that the loss of enzyme activity was associated with the loss of cations from the particles. However, the need for more ATP and the appearance of some inhibitory substance(s) after ageing of microsomes showed that the inactivation process was more complex. More ATP may be needed by the

liver-enzyme system after ageing of the microsomes because of a loss of ATP through an increased adenosine triphosphatase activity. This idea is supported by the fact that sodium fluoride, which by itself had no definite power to reactivate old microsomes, enhanced the synthetic activity of the enzyme system in the presence of high concentrations of ATP. However, the effect of sodium fluoride could be observed only with very old preparations of microsomes, whereas the need for more ATP and Mg^{2+} (or Mn^{2+}) ions appeared within a few days of storage at -15°. With microsome preparations at least up to 8 days old sodium fluoride had no effect, even in the presence of high concentrations of ATP (Table 7). This suggests an additional mechanism for loss of ATP, possibly through chelation with some substance (e.g. Ca^{2+} ions) released from the microsomes. Ca^{2+} ions in a concentration of mM inhibited sterol synthesis by 75 %, but this was only partly counteracted by an increased concentration of Mg^{2+} ions. It is, therefore, difficult to decide whether the activating effect of Mg^{2+} or Mn^{2+} ions on microsomes may be attributed solely to a replacement of metal ions lost, or to a neutralizing of inhibitors released from the particles during ageing; both mechanisms may operate. The results shown in Table 6 indicate that the inhibitory effect of the supernatant of an aged microsomal preparation was counteracted by the reactivating system.

A practical consequence of the present study is that suspensions of microsomes kept frozen at -15° for at least as long as 4 months may be used satisfactorily in a cholesterol-synthesizing enzyme system provided that the concentration of Mg^{2+} ions is raised to 5 mM and that of ATP to 7.5 mM (cf. 'standard incubation mixture' under Methods); by the inclusion of sodium fluoride (10 mM) the synthetic activity is raised further so that the results obtained cannot be distinguished from those obtained with a system in which fresh microsomes are employed.

SUMMARY

1. The enzyme system from rat liver, consisting of a soluble protein fraction and microsomes, and which synthesizes cholesterol from mevalonic acid, was investigated for stability of the microsomal enzymes.

2. Microsomes kept frozen at -15° for a few weeks to a few months are unable to catalyse sterol synthesis when combined with the soluble enzymes under conditions optimum for an enzyme system containing fresh microsomes.

3. Inactivation of fresh microsomes can be induced rapidly by incubation at 37°.

4. Partial reactivation of microsomes stored at -15°, or of those 'aged' at 37°, can be achieved by

Table 7. *Effects of adenosine triphosphate and of sodium fluoride on enzyme system containing microsomes 8 days old*

Each flask contained the standard incubation mixture with microsomes stored at -15° for 8 days. Additions were as shown.

Additions (μ moles)	Specific activity of sterol digitonides (counts/min. at infinite thickness)
None	5 156
ATP (20)	13 997
NaF (40)	5 238
ATP (20) + NaF (40)	12 324

the addition of Mn^{2+} ions (mM) or by an increased concentration of Mg^{2+} ions (5 mM). The sterol-synthesizing system has an absolute requirement for Mg^{2+} or Mn^{2+} ions.

5. Enzyme systems containing old preparations of microsomes have also a requirement for adenosine triphosphate higher than those containing fresh microsomes. Sodium fluoride, which has no effect on fresh preparations, enhances the stimulating effects of high concentrations of adenosine triphosphate on old preparations of microsomes.

6. During ageing, substances inhibiting sterol synthesis appear in the microsomal preparations.

7. Microsome preparations as old as four months can be fully reactivated by a suitable combination

of Mg^{2+} ions, adenosine triphosphate and sodium fluoride.

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The Metabolism of [16- ^{14}C]Oestrone *in vitro*

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Since the original demonstration by Zondek (1934) that liver mince rapidly inactivates oestrogens, many workers have studied the metabolism of oestrone and oestradiol *in vitro*, employing loss of biological activity to follow hormone-degradation (Heller, 1940; Engel & Rosenberg, 1945; Levy, 1947; De Meio, Rakoff, Cantarow & Paschkis, 1948; Pearlman & De Meio, 1949). Ryan & Engel (1953), by means of countercurrent distribution and fluorometric analysis, found that after incubation of these two natural oestrogens with rat-liver slices over 50 % of the starting material was converted into unknown metabolites. More recent experiments with [^{14}C]oestrogens *in vivo* have supported these findings. Thus Valcourt, Thayer, Doisy, Elliott & Doisy (1955), on administering [16- ^{14}C]oestrone to rats, found that even after enzymic hydrolysis of conjugates only one-third of the ^{14}C excreted in bile and urine was extractable with ether, a solvent in which steroid oestrogens are readily soluble. Similarly, Beer & Gallagher (1955) showed that extraction with ether failed to remove all the radioactivity from hydrolysed urine of human patients injected with [^{14}C]oestrone or [^{14}C]oestradiol, and, in perfusion experiments with radioactive oestrone, Wotiz, Ziskind & Ringler (1958) observed a large amount of chloroform-insoluble material containing ^{14}C in the hydrolysed dialysates of rat plasma.

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Very little is known about the nature of the inactivation products of oestrogens, but there is evidence that oestrone and oestradiol are attacked by an oxidative enzyme system. Thus Heller (1940) found that cyanide inhibited the inactivation of oestrogens by liver, suggesting that an oxidase was involved, and in support of this theory Westerfeld (1940) and Graubard & Pincus (1941) showed that oxidases from plant sources were capable of acting on oestrogens. Levy (1947), using rat-liver homogenates, also showed inhibition by cyanide as well as by azide and carbon monoxide, and suggested that the cytochrome system was involved. He found that inactivation of oestradiol was greatly reduced under anaerobic conditions, and this was confirmed by De Meio *et al.* (1948), who, however, found very little inhibition of oestrogen-inactivation by cyanide or azide and none by malonate, iodoacetate or fluoride. In all these experiments very little conjugation was observed and the results which were obtained by bioassay methods give no indication about the extent to which the original steroid molecule had been altered.

The advent of isotope-labelled oestrogens, however, made the solution of this problem more feasible, and the present investigation was therefore undertaken to determine whether the water-soluble ether-insoluble metabolites observed *in vivo* are also formed *in vitro* and, if so, to try and discover the nature of these products and the effect of enzyme inhibitors on their formation.