thyroid hormones in a given animal is determined by the rapidity with which they leave the blood and reach their intracellular sites of action. The study of thyroid-hormone transport in birds has therefore been valuable in revealing the physiological significance of similar data previously obtained in mammals.

SUMMARY

1. The similar potencies of L-thyroxine and 3:5:3′-tri-iodo-L-thyronine in the chicken have been further studied by comparing the overall rate of metabolism of the two thyroid hormones in both mammals and birds and by comparing binding of thyroxine by serum proteins in man, chicken and duck.

2. Thyroxine and tri-iodothyronine have indistinguishable rates of disappearance from the whole body in the chicken with half-lives of 22·5 ± 1 hr.

3. Thyroxine-binding protein as found in human serum is absent from, or present only in small amounts in, chicken and duck sera. Both endogenously labelled and exogenously labelled thyroid hormones are mostly bound to albumin in the sera of these birds.

4. Quantitative studies show that both affinity and capacity of chicken serum to bind thyroxine are lower than those found for human serum. The major thyroxine-binding protein fractions in human serum, Cohn fraction IV-4 and prealbumin, bind thyroxine about three to four times more firmly than tri-iodothyronine. Human-, chicken- and duck-serum-albumin fractions bind both hormones with almost identical intensities.

5. It is concluded that the difference in the response of birds and mammals to tri-iodothyronine, as compared with thyroxine, is due to a fundamental difference in the binding of thyroid hormones to avian-serum proteins and mammalian-serum proteins respectively.

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Inhibition of Aconitase by Glyoxylate plus Oxaloacetate

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Previous experiments showed that glyoxylate incubated with oxaloacetate in homogenates of rat liver and other animal tissue (D'Abramo, Romano & Ruffo, 1957*a*, *b*; 1958) produced a complete inhibition of citrate oxidation. The inhibition was explained by supposing that the two substances might react together to form a compound of six carbon atoms, possibly oxalomalic acid, which, even at very low concentration, could inhibit citrate oxidation by competition with aconitase or *iso*-citric dehydrogenase. Of these, aconitase was the first enzyme tested for the possibility that it could be inhibited by a condensation compound formed during the incubation of glyoxylate with oxaloacetate.

The results of these experiments show that a complete inhibition of citrate formation from *cis*-aconitate occurred in both crude and purified

aconitase preparations, when the incubation was in the presence of oxaloacetate plus glyoxylate. Evidence is also presented that oxaloacetate and glyoxylate react together non-enzymically in the presence of magnesium to produce an inhibitor of aconitase.

EXPERIMENTAL

Preparation of crude aconitase. The method of Johnson (1939) was followed, rat liver being used instead of pigeon-breast muscle. The liver was cut into small pieces, ground in a mortar at 0° and mixed with 5 vol. of 0·1 M-phosphate buffer (17·8 g. of $Na_2HPO_4,2H_2O$ and 20 ml. of N-HCl to 1000 ml. of water); pH was 7·4. The mixture was centrifuged at 0° (4000 rev./min. for 10 min.) and the supernatant was diluted (1:10) with 0·1 M-phosphate buffer containing 0·9% of KCl. A sample (3 ml.) of the clear extract was used for the activity determinations.

Purification of aconitase from pig heart. The method of Anfinsen (1955), based on ethanol fractionation of pig-heart homogenate, followed by fractionation with ammonium sulphate, was used. An 18-fold-purified preparation was obtained in the form of a pale-pink amorphous precipitate, which was dissolved before use in 0·1 M-phosphate buffer (as described above) containing 0·05 M-sodium citrate; pH was 8·2. These solutions contained 2–3 mg. of protein/ml. and approx. 50 units/mg. Portions (0·1–0·2 ml.) of these solutions were used for the single determinations.

Determination of aconitase activity. In crude extracts the activity was determined by incubating 10 μmoles of *cis*-aconitate (Sigma Chemical Co., St. Louis, Mo., U.S.A.) with 3 ml. of diluted rat-liver extract and adjusting the volume to 4 ml. with the phosphate buffer–KCl. The time of incubation at 38° was 1 hr., unless otherwise indicated, and the reaction was stopped by adding 3 ml. of freshly prepared 5% (w/v) tungstic acid.

With the purified preparations the assay method suggested by Anfinsen (1955) was followed. A volume (0·5 ml.) of 0·2 M-*cis*-aconitate solution (100 μmoles) was incubated with 0·1–0·2 ml. of enzyme diluted to a final volume of 4 ml. with 0·1 M-phosphate buffer, pH 7·4. The incubation, carried out at 38° for 30 min., was stopped by adding 0·5 ml. of 30% (w/v) trichloroacetic acid.

Citrate determination. Citrate was determined in the tungstic acid filtrates by the method of Ettinger, Goldbaum & Smith (1952), which was most suitable for the determination of small amounts of citrate, and in the trichloroacetic acid filtrates by the method described by Stern (1957).

Where indicated, the enzyme preparations were incubated with glyoxylate or oxaloacetate (L. Light and Co. Ltd., Colnbrook, Bucks) or both. The concentrations used do not interfere in the determination of citrate.

Absorption spectra and chromatographic determination of 2:4-dinitrophenylhydrazones. Two portions (0·1 and 0·4 ml.) of the trichloroacetic acid filtrates, after dilution to 2 ml. with water, were treated with 0·5 ml. of 0·2% (w/v) 2:4-dinitrophenylhydrazine. To the first portion, 2·5 ml. of 2 N-NaOH was added for the determination of absorption spectra. The readings were done with a Beckman spectrophotometer between 400 and 600 mμ, against a blank of the phenylhydrazine, water and NaOH soln. The second was treated with formaldehyde according to Cavallini & Mondovì (1957), extracted with ether, dried *in vacuo* and redissolved in 0·8 ml. of aq. N-NH_3 soln.–$CHCl_3$ (1:1, v/v). A portion (0·05 ml.) of the aqueous phase was spotted on Schleicher und Schüll (Dassel, Germany) no. N. 2043*b* filter paper, and chromatographed, by descending chromatography, for 14 hr. in butan-1-ol saturated with aq. 3% (v/v) NH_3 soln. The spots were developed with 2 N-NaOH. The results of these experiments are reported in Table 3. Fig. 3 shows the chromatogram of the phenylhydrazones obtained from the filtrates of the experiment with purified aconitase (Table 2, Expt. 3).

RESULTS

Inhibition of crude aconitase. Rat-liver extracts diluted in 0·1 M-phosphate buffer, pH 7·4, containing 0·9% of KCl were incubated in a final volume of 4 ml. with 10 μmoles of *cis*-aconitate in the presence and in the absence of different amounts of glyoxylate and oxaloacetate, added alone and together, as reported in Table 1.

The results showed that whereas the addition of either oxaloacetate or glyoxylate alone did not produce any effect on the formation of citrate from

Table 1. *Inhibition of crude aconitase by glyoxylate plus oxaloacetate*

A portion (3 ml.) of rat-liver extract diluted (1:50) with phosphate buffer–KCl, pH 7·4, was used. Additions: *cis*-aconitate (10 μmoles), glyoxylate and oxaloacetate as indicated. Final volume, 4 ml.; incubation, 60 min.; temp. 38°. For deproteinization 3 ml. of 5% (w/v) tungstic acid was used.

Expt.	Additions	Citrate found (μmoles)	Inhibition (%)
1	*cis*-Aconitate	7·7	—
	cis-Aconitate + 4 μmoles of glyoxylate	7·7	0
	cis-Aconitate + 8 μmoles of oxaloacetate	7·7	0
	cis-Aconitate + oxaloacetate + glyoxylate	0·4	94
2	*cis*-Aconitate	8·4	—
	cis-Aconitate + 5 μmoles of glyoxylate	8·2	2
	cis-Aconitate + 5 μmoles of oxaloacetate	8·1	3
	cis-Aconitate + glyoxylate + oxaloacetate	0	100
3	*cis*-Aconitate	7·1	—
	cis-Aconitate + 2 μmoles of glyoxylate	7·0	1
	cis-Aconitate + 2 μmoles of oxaloacetate	7·0	1
	cis-Aconitate + oxaloacetate + glyoxylate	0·9	87

cis-aconitate, when these substances were added together almost a complete inhibition of the reaction occurred. Since the inhibition was 87 % also with amounts of glyoxylate and oxaloacetate as low as 2 μmoles/flask (0·5 mM), the effect of lower concentrations of glyoxylate and of oxaloacetate was investigated. The results in Fig. 1 show that approximately 50 % inhibition was obtained for the addition of very low amounts (0·1 μmole; 25 μM) of either oxaloacetate (to the incubation mixtures containing glyoxylate) or glyoxylate (to the incubation mixtures containing oxaloacetate). The inhibition reached maximal values with amounts of either glyoxylate or oxaloacetate between 0·4 and 0·6 μmole/flask (0·1–0·15 mM).

Another point investigated was the effect of the time of incubation on the rate of inhibition. The curves of Fig. 2 (*a*) show that in the presence of glyoxylate and oxaloacetate the formation of citrate from *cis*-aconitate was inhibited at the beginning of the incubation; those of Fig. 2 (*b*) show that between only 4 and 6 min. of incubation the inhibition was already evident.

Inhibition of purified aconitase. The results reported in Table 2 were obtained with a purified aconitase preparation from pig heart according to the method of Anfinsen (1955). They show that

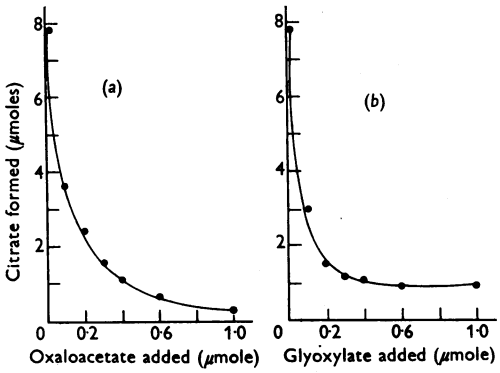

Fig. 1. Inhibition of aconitase by oxaloacetate and glyoxylate. (*a*) Incubation mixtures contained 4 μmoles of glyoxylate and oxaloacetate as indicated. (*b*) Incubation mixtures contained 4 μmoles of oxaloacetate and glyoxylate as indicated. With both (*a*) and (*b*) incubation was for 1 hr. at 38°, with 10 μmoles of *cis*-aconitate and 3 ml. of diluted (1:50) liver extract in 0·1 M-phosphate buffer, pH 7·4, containing 0·9 % of KCl. Final volume, 4 ml. For deproteinization 3 ml. of freshly prepared 5 % (w/v) tungstic acid was used.

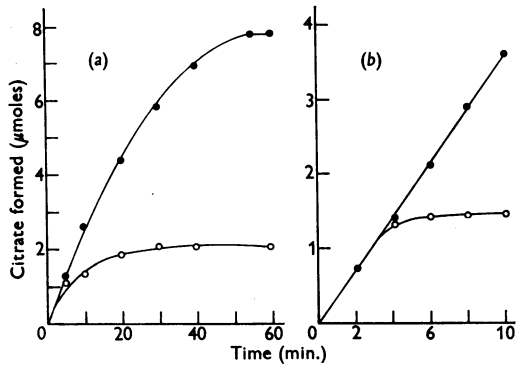

Fig. 2. Rate of inhibition of aconitase. ●, Incubation mixtures contained 3 ml. of diluted (1:50) liver extract in 0·1 M-phosphate buffer containing 0·9 % of KCl, and 10 μmoles of *cis*-aconitate; ○, incubation mixtures, contained in addition 4 μmoles each of oxaloacetate and glyoxylate. Final volumes, 4 ml. The reaction was stopped by the addition of 3 ml. of 5 % (w/v) tungstic acid at the times indicated. (*a*) Incubation was at 38° for 1 hr.; (*b*) incubation was at 38° for 10 min.

Table 2. *Inhibition of purified aconitase by glyoxylate plus oxaloacetate*

Enzyme was present as indicated. Additions: *cis*-aconitate, 100 μmoles; oxaloacetate, 5 μmoles if present; glyoxylate as indicated; 0·1 M-phosphate buffer, pH 7·4. Incubation was for 30 min. at 38°; final volume, 4 ml. For deproteinization 0·5 ml. of 30 % (w/v) trichloroacetic acid was used.

Expt.	Enzyme: Vol. (ml.)	Enzyme: Concn. (mg. of protein/ml.)	Additions	Citrate formed (μmoles)	Inhibition (%)
1	0·2	3·1	Nil	42	—
			Oxaloacetate	41	2
			Glyoxylate (50 μmoles)	40	4
			Oxaloacetate + glyoxylate	2	95
2	0·1	2·0	Nil	35	—
			Oxaloacetate	35	0
			Glyoxylate (5 μmoles)	35	0
			Oxaloacetate + glyoxylate	0	100
3	0·1	3·0	Nil	28	—
			Oxaloacetate	27	3
			Glyoxylate (5 μmoles)	28	0
			Oxaloacetate + glyoxylate	0	100

with this preparation the addition of small amounts (5 μmoles; 1·25 mM) of glyoxylate plus oxaloacetate produced almost a complete inhibition of citrate formation from *cis*-aconitate.

To investigate the mechanism of the inhibition, portions of the trichloroacetic acid filtrates of the last experiment (Table 2, Expt. 3) were taken for chromatographic assay of the phenylhydrazones. Results presented in Fig. 3 show the spots obtained from the different filtrates after incubation for 60 min. No phenylhydrazone spots appeared in sample no. 1, corresponding to the incubation of the enzyme with *cis*-aconitate, whereas the characteristic spots of glyoxylate and oxaloacetate were shown in nos. 2 and 3. A new spot, not far from the starting point, whose R_F was different from all the others, appeared in the filtrate from Expt. 4, where oxaloacetate and glyoxylate were incubated together and produced the inhibition of aconitase. The position of this new spot on the chromatogram was similar to that indicated by Cavallini & Frontali (1954) for the phenylhydrazone of oxalosuccinic acid (R_F approx. 0·07).

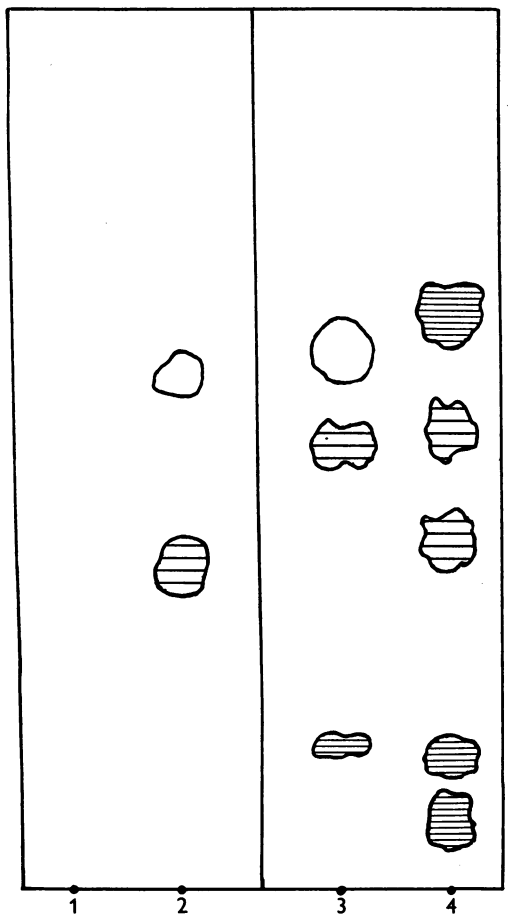

Fig. 3. Chromatogram of the phenylhydrazones obtained after incubation of oxaloacetate and glyoxylate with purified aconitase. 1, Enzyme only; 2, enzyme plus glyoxylate; 3, enzyme plus oxaloacetate; 4, enzyme plus oxaloacetate and glyoxylate. Incubation mixtures contained: 0·1 ml. of enzyme solution; 5 μmoles each of oxaloacetate and glyoxylate in 0·1 M-phosphate buffer, pH 7·4, to make the volume 4 ml. Incubation was at 38° for 30 min.; for deproteinization 0·5 ml. of trichloroacetic acid was used.

Incubation of glyoxylate plus oxaloacetate without the enzyme. The preceding results show that during the incubation of a mixture containing *cis*-aconitate, oxaloacetate, glyoxylate and aconitase, a new substance forming a phenylhydrazone appeared. It is possible that this substance formed from the reaction of oxaloacetate plus glyoxylate is responsible for the inhibition of aconitase. Since this reaction appeared to occur in the purified aconitase preparation without condensing enzymes, the possibility that this compound may be formed by a spontaneous (not enzymic) reaction of oxaloacetate with glyoxylate was investigated. Glyoxylate and oxaloacetate were incubated in Krebs phosphate saline free from Ca^{2+} ions (Umbreit, Burris & Stauffer, 1951), water, or 0·1 M-phosphate buffer, both in the presence and the absence of Mg^{2+} ions. In other experiments Mn^{2+} ions were substituted for Mg^{2+} ions. The chromatographic results (Table 3) showed that either oxaloacetate or glyoxylate after incubation for 1 hr. produced, usually, two spots of rather constant R_F. The spot with the lowest R_F (0·14–0·16) is the hydrazone of oxaloacetate, the second and the third are the hydrazones of pyruvate. This keto acid can give two spots, the *syn*- and *anti*-forms (Cavallini & Frontali, 1954), and this may account for our finding in the experiment with enzyme, where probably more oxaloacetate was converted into pyruvate (Fig. 3 and Table 3, Expt. 1). The R_F values of glyoxylate hydrazones were reasonably similar in the different media investigated, and comparable with the results of Cavallini & Frontali (1954), who also found two spots.

In the experiments in which oxaloacetate and glyoxylate were incubated together, a new hydrazone was formed whose R_F was different from those of either oxaloacetate or glyoxylate. This new substance appeared in the experiment with enzyme (Table 3, Expt. 1) and also in experiments where the incubation was carried out in Krebs phosphate saline (Expt. 2), in water (Expt. 4) and in phosphate buffer (Expt. 7), in which Mg^{2+} ions were present. It was not present in the media without Mg^{2+} ions (Expts. 3, 5 and 6). It should be noted that the position of this new spot on the chromatograms was near to the starting line (R_F 0·07), and appeared to be similar to that attributed by

Table 3. *Chromatographic separation of the phenylhydrazones of glyoxylate and oxaloacetate*

Glyoxylate (5 μmoles) or oxaloacetate (5 μmoles) or both were incubated at 38° for 1 hr. in 4 ml. of the media indicated below. A volume (0·5 ml.) of 30% (w/v) trichloroacetic acid was then added and after treatment with 2:4-dinitrophenylhydrazine portions corresponding to approx. 0·05 μmole of the original keto acids were spotted on papers. Results given are the R_F values of the single spots developed with 2N-NaOH In Expts. 6 and 7 portions from mixtures in which oxaloacetate or glyoxylate was the sole substrate have not been chromatographed.

		Substrates incubated		
Expt.	Incubation media	Glyoxylate	Oxaloacetate	Oxaloacetate plus glyoxylate
1	0·1M-Phosphate buffer, pH 7·4, and enzyme	0·36, 0·58	0·15, 0·58, 0·61	0·06, 0·14, 0·40 0·51, 0·64
2	Krebs phosphate saline (approx. 14 μg.atoms of Mg^{2+} ions)	0·38, 0·60	0·16, 0·57	0·07, 0·17, 0·40 0·57, 0·66
3	Water	0·31, 0·52	0·14, 0·42	— 0·14, 0·31 0·38, 0·50
4	Water plus 100 μg.atoms of Mg^{2+} ions	0·31, 0·52	0·14, —	0·05, 0·12, 0·30 — 0·52
5	Water plus 100 μg.atoms of Mn^{2+} ions	0·32, 0·55	0·14, 0·43	— 0·14, 0·32 0·35, 0·53
6	0·1M-Phosphate buffer, pH 7·4	— —	— —	— 0·15, 0·47 0·54, 0·66
7	0·1M-Phosphate buffer plus 100 μg.atoms of Mg^{2+} ions	— —	— —	0·07, 0·16, 0·44 0·53, 0·64

Cavallini & Frontali (1954) to oxalosuccinic acid, a tricarboxylic acid with six carbon atoms.

Moreover, the absorption spectra of the dinitrophenylhydrazones, obtained after similar incubation experiments, showed (Fig. 4) that a fall of absorption at 450 mμ occurred in the samples corresponding to the incubation of oxaloacetate plus glyoxylate in water and in the presence of Mg^{2+} ions (Fig. 4*d*). These results were not obtained in the absence of Mg^{2+} ions (Fig. 4*b*). Comparison of the curves (*b*) and (*d*) with the results reported in Table 3 shows that the fall of the absorption spectra, after the incubation of oxaloacetate plus glyoxylate, occurred only in the same experimental conditions in which the additional spot was detectable, i.e. in the presence of Mg^{2+} ions. Similar results, not reported in the text, were obtained also by incubating oxaloacetate plus glyoxylate in Krebs phosphate saline, and in phosphate buffer. The fall in absorption was observed only in the Krebs phosphate saline, which contains Mg^{2+} ions.

Effect of Mg^{2+} *ions on the inhibition of aconitase.* To confirm that the additional chromatographic spot appearing in the preceding experiments, when oxaloacetate and glyoxylate were incubated together in the presence of Mg^{2+} ions, was related to the formation of the inhibitor, crude aconitase was incubated in phosphate buffer in the absence of Mg^{2+} ions, with the concentration of oxaloacetate reduced to 0·06 μmole/flask to decrease the inhibition approximately to 50%. To similar incubation mixtures, increasing concentrations of Mg^{2+} ions were added, as indicated in Fig. 5, and citrate was determined after incubation for 1 hr.

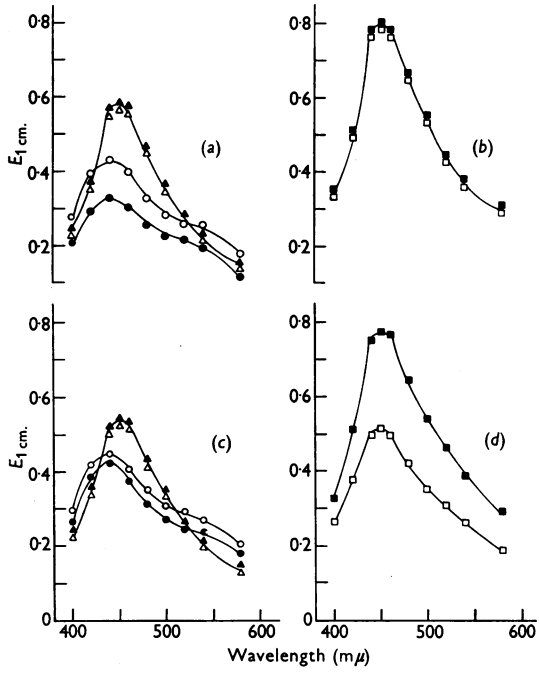

Fig. 4. Absorption curves of the phenylhydrazones obtained before (filled-in points) and after (unfilled points) incubation with 5 μmoles of oxaloacetate (○) or of glyoxylate (△) or glyoxylate plus oxaloacetate (□). Final volume, 4 ml.; incubation was at 38° for 1 hr. A volume (0·5 ml.) of 30% (w/v) trichloroacetic acid was added at the end of the incubation. (*a*) and (*b*) Incubation in water; (*c*) and (*d*) incubation in water plus 100 μg.-atoms of Mg^{2+} ions.

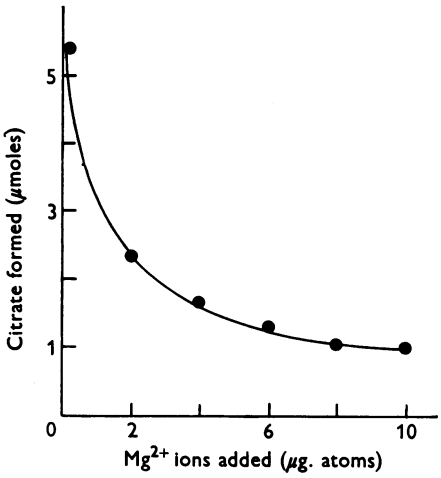

Fig. 5. Effect of Mg^{2+} ions on the inhibition of aconitase. The incubation mixture contained 3 ml. of diluted (1:50) liver extract in 0·1 M-phosphate buffer containing 0·9% of KCl, 0·06 μmole of oxaloacetate, 4 μmoles of glyoxylate and $MgCl_2$ as indicated, in the final volume of 4 ml. Incubation was at 38° for 1 hr.; for deproteinization 3 ml. of freshly prepared 5% (w/v) tungstic acid was used.

The results showed that the inhibition increased by increasing the concentration of Mg^{2+} ions until it reached the value of approximately 90%, at the concentration of 10 μg.atoms of Mg^{2+} ions/flask.

DISCUSSION

Results presented in this paper seem to confirm earlier ones (D'Abramo *et al.* 1958), according to which oxaloacetate and glyoxylate may react together to form a compound which inhibits citrate oxidation. The results show that aconitase was strongly inhibited by the incubation of glyoxylate plus oxaloacetate. Its activity was reduced between 75 and 100% irrespective of the degree of purity. Chromatographic analysis of the incubation mixtures showed the formation of a substance forming a hydrazone different from that of oxaloacetate and glyoxylate. Since the R_F of this new spot appeared to be similar to that of oxalosuccinic acid, it seems possible that the new spot observed in our chromatograms is another tricarboxylic acid with six carbon atoms, oxalomalic acid, which theoretically could result from the reaction of glyoxylate plus oxaloacetate. Evidence has been obtained that oxaloacetate and glyoxylate may react together without enzyme in media containing Mg^{2+} ions to produce an inhibitor of aconitase.

In conclusion, both the experiments on aconitase inhibition and those showing chromatographic and spectrophotometric evidence of the formation of the condensation compound seem to explain our previous results (D'Abramo *et al.* 1958), which showed the inhibition of citrate oxidation produced by the incubation with glyoxylate and oxaloacetate. A new compound is formed from the spontaneous reaction of glyoxylate plus oxaloacetate, and has been identified as the aconitase inhibitor. If this compound is a tricarboxylic acid with six carbon atoms (oxalomalic acid) it might inhibit citrate oxidation by competition with citrate for aconitase. The chemical nature of this compound is under investigation.

SUMMARY

1. Crude or purified aconitase preparations were inhibited after incubation in the presence of glyoxylate and oxaloacetate. Inhibition was demonstrated with amounts as low as 0·1 μmole of either glyoxylate or oxaloacetate in the presence of 4 μmoles of oxaloacetate or glyoxylate respectively.

2. Chromatographic evidence is given of the presence of a new compound formed after incubation of the purified enzyme with glyoxylate and oxaloacetate.

3. The presence of the same compound was chromatographically detected after incubation of glyoxylate and oxaloacetate in the presence of magnesium, but without enzyme. Spectrophotometric evidence for the disappearance of glyoxylate or oxaloacetate or both is given.

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