REFERENCES

Ashby, W. (1951). *J. nerv. ment. Dis.* **114**, 391.
Bailey, K. (1942). *Biochem. J.* **36**, 121.
Binkley, F. & Olson, C. K. (1950). *J. biol. Chem.* **186**, 725.
Dubois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **150**, 185.
Epelbaum, S. E., Sheves, G. & Kobylin, A. A. (1949). *Biochemistry, Leningr.*, **14**, 107.
Feldberg, W. & Mann, T. (1945). *J. Physiol.* **103**, 28*P*.
Fleischhacker, H. H. (1938). *J. ment. Sci.* **84**, 947.
Fleischhacker, H. H. & Yates, E. D. (1936). *Chem. and Ind.* **14**, 943.
Giri, K. V. & Datta, N. C. (1936). *Biochem. J.* **30**, 1089.
Gordon, J. J. (1950). *Biochem. J.* **46**, 96.
Gore, M. (1952). *Biochem. J.* **50**, 18.
King, E. J. (1931). *Biochem. J.* **25**, 799.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
Landow, H., Kabat, E. A. & Newman, W. (1942). *Arch. Neurol. Psychiat., Chicago*, **48**, 518.
Macfarlane, M. G., Patterson, L. M. & Robison, R. (1934). *Biochem. J.* **28**, 720.
Meyerhof, O. & Wilson, J. R. (1948). *Arch. Biochem.* **17**, 153.
Naidoo, D. & Pratt, O. E. (1951). *J. Neurol. Psychiat.* **14**, 287.
Naidoo, D. & Pratt, O. E. (1952). *J. Neurol. Psychiat.* **15**, 164.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Reis, J. (1937). *Enzymologia*, **2**, 110.
Richter, D. & Hullin, R. P. (1951). *Biochem. J.* **48**, 406.
Scharrer, E. & Sinden, J. (1949). *J. comp. Neurol.* **91**, 331.
Vondracek, V. (1927). *Biochem. Z.* **191**, 88.
Wolf, A., Kabat, E. A. & Newman, W. (1943). *Amer. J. Path.* **19**, 423.

Biosynthesis of Choline in the Seedling of the Chick-pea (*Cicer arietinum*)

By K. AHMAD and M. A. KARIM
Biochemistry and Nutrition Laboratory, University of Dacca, East Pakistan

(*Received* 23 *April* 1953)

The problem of methyl-group biosynthesis in the plant has not received as much attention as in the animal. Amongst important papers on the subject are those showing the formation of the methyl groups of hordenine and choline from formate by sprouting barley (Kirkwood & Marion, 1951) and of the methyl carbon of nicotine from formate and the methyl group of methionine by *Nicotiana rustica* L. (Brown & Byerrum, 1952). Barrenscheen & Pany (1941) and Barrenscheen & Valyi-Nagy (1942) observed the methylation of guanidoacetic acid to give creatine by 7-day-old wheat seedlings and also found that added methionine increased creatine synthesis sixfold to eightfold. They observed creatine synthesis to be an aerobic process where addition of hydrogen acceptors like cystine could not replace oxygen. They also noted that the transfer of the methyl group of methionine was accompanied by oxidation of the sulphur. Steensholt (1946), however, found that etiolated wheat germs were unable to use methionine for the methylation of ethanolamine to choline.

This paper describes experiments showing how the biological elaboration of the important methyl compound, choline, by the germinating seedlings of *Cicer arietinum* is influenced by certain inorganic salts, vitamins and by some organic compounds already known to be concerned with the biochemistry of methyl groups elsewhere.

EXPERIMENTAL

Technique of germination. 16–20 g. of seeds of *C. arietinum* were soaked in distilled water or in an aqueous solution of the test substance (hereafter designated as 'germinating fluid') for 12 hr. and then allowed to germinate on a sand bed in large baking dishes at room temperature (25–28°). At the end of the germination period, the seedlings, after removal of the seed coat, were dried and choline was determined.

Determination of choline. An adaptation of the methods described by Luecke & Pearson (1944) and McIntire, Sweigert and Elvehjem (1944) was used. The finely minced seedlings (1 g.) were autoclaved with 10 ml. of 3 N-HCl for 2 hr. at 15 lb./sq. in. The hydrolysate after neutralization with NaOH was passed through a column of Decalso at a rate of 6 to 10 drops/min. The adsorbed choline was then eluted with two 10 ml. portions of 5% NaCl. 5 ml. of 2% ammonium reineckate in methanol were added to the eluate, which was kept at −4° for 24 hr. to allow complete precipitation of choline reineckate. The precipitate was then collected on an asbestos pad and washed three times with 2 ml. portions of ice-cold ethanol. Air was drawn through the pad until the precipitate was dry. The precipitate was then washed down with acetone and the washings were made up to 25 ml. The colour due to choline reineckate was measured in a photoelectric (Klett-Summerson) colorimeter. A standard, with 4 mg. of choline chloride, and a reagent blank were run with every determination.

Each set of experiments described below was run five times, and every determination done in triplicate. No variation over 5% was noticed and the results presented are average values.

RESULTS

During germination, the choline contents of the seedlings (Table 1, column 1) increased gradually to a maximum value of 3·78 mg. (expressed as choline chloride) per gram of the seedling (dry weight) after 96 hr. of germination and then declined. The same results were obtained in either light or darkness.

Table 1. *Distribution of choline in the cotyledon and growing part of the embryo (shoot plus radicle) of* Cicer arietinum *at various times after germination*

(The seeds were soaked in distilled water for 12 hr. and then allowed to germinate on a sand bed.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.)	Choline chloride (% of total) in	
		Cotyledons	Shoot plus radicle
0	1·81	100	—
24	2·35	96·2	3·8
48	2·97	82·0	18·0
72	3·51	65·5	34·5
96	3·78	49·0	51·0
120	3·58	35·0	65·0
144	2·89	25·0	75·0

Table 1 also shows the distribution of choline between the cotyledon and the growing part of the embryo (shoot and radicle) with the progress of germination. The amount in the latter increased with a concomitant decrease in the former.

Table 2 shows the effects of certain salts on the choline contents of the seedling with the progress of germination. While sodium chloride had no stimulatory effect, 0·01 M solutions of potassium chloride, potassium nitrate, ammonium chloride and ammonium nitrate stimulated choline biosynthesis considerably and after 96 hr. produced maximum increases of 10·5, 18·5, 15·9 and 27·6 %, respectively, over the control.

Table 3 shows the effects of three water-soluble vitamins, thiamine, riboflavin and ascorbic acid, added as 0·025 % (w/v) aqueous solutions. While ascorbic acid depressed choline biosynthesis by 22·9 %, riboflavin and thiamine stimulated it by 10·5 %.

Table 4 shows the effects of DL-methionine (0·1 %, w/v), creatinine (0·1 %, w/v), acetone (1 %, v/v) and methanol (1 %, v/v) which stimulated synthesis by 31·6, 29·2, 9·2 and 4·2 %, respectively, over the control.

Table 2. *Effect of certain inorganic salts on the biosynthesis of choline in the germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in 0·01 M salt solutions and then allowed to germinate on a sand bed. The figures in parentheses represent the variation from the corresponding value of the control experiment. A negative value indicates depression, a positive value stimulation, of biosynthesis.

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was					
	Water (control)	NaCl	KCl	KNO_3	NH_4Cl	NH_4NO_3
0	1·82	1·82	1·82	1·82	1·82	1·82
24	2·38	2·38	2·66	3·00	2·90	3·22
48	2·98	2·93	3·42	3·63	3·63	3·90
72	3·50	3·21	3·80	4·40	3·97	4·50
96	3·80	3·62 (−4·7 %)	4·20 (10·5 %)	4·50 (18·4 %)	4·40 (15·9 %)	4·85 (27·6 %)
120	3·50	3·22	3·97	4·20	4·20	4·50
144	2·88	3·22	3·63	3·81	3·97	4·10
168	2·44	3·10	3·52	3·75	3·21	3·80

Table 3. *Effect of certain water-soluble vitamins on the biosynthesis of choline in the germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in aqueous solutions of the vitamins (0·025 %, w/v) before germination on a sand bed. The meaning of the figures in parentheses is given in Table 2.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was			
	Water (control)	Thiamine	Riboflavin	L-Ascorbic acid
0	1·82	1·82	1·82	1·82
24	2·38	2·62	2·38	2·07
48	2·98	2·96	2·93	2·67
72	3·50	3·82	3·63	2·75
96	3·80	4·20 (10·5 %)	4·20 (10·5 %)	2·93 (−22·9 %)
120	3·50	4·11	3·81	2·67
144	2·88	3·43	3·42	2·68

Table 4. *Effect of certain organic compounds on the biosynthesis of choline in germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in an aqueous solution of the test substance and then allowed to germinate on a sand bed. The meaning of the figures in parentheses is given in Table 2.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was				
	Water (control)	DL-Methionine (0·1%, w/v)	Creatine (0·1%, w/v)	Acetone (1·0%, v/v)	Methanol (1·0%, v/v)
0	1·82	1·82	1·82	1·82	1·82
24	2·38	2·75	2·64	2·68	2·52
48	2·98	3·51	3·22	3·43	2·96
72	3·50	4·50	4·20	3·91	3·90
96	3·80	5·00 (31·6%)	4·91 (29·2%)	4·15 (9·2%)	3·96 (4·2%)
120	3·50	4·80	4·75	3·97	3·63
144	2·88	4·20	3·92	2·93	3·43
168	2·44	3·90	3·22	2·52	2·82

Table 5. *Effect of certain organic compounds on the biosynthesis of choline in the germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in the aqueous solutions of the test substances and then allowed to germinate on a sand bed. The meaning of the figures in parentheses is given in Table 2.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was				
	Water (control)	Nicotinamide (0·1%, w/v)	Homocystine (0·1%, w/v)	Nicotinamide (0·1%, w/v) + creatinine (0·1%, w/v)	Nicotinamide (0·1%, w/v) + acetone (1%, v/v)
0	1·81	1·81	1·81	1·81	1·81
24	2·38	2·00	2·38	2·38	2·43
48	2·99	2·38	2·68	2·68	2·64
72	3·50	2·68	2·92	3·02	3·22
96	3·80	2·69 (−29·2%)	2·98 (−21·6%)	3·22 (−15·2%)	3·80 (0%)
120	3·50	2·00	2·98	3·20	3·22
144	2·88	2·60	2·96	2·88	2·88
168	2·44	1·50	2·52	2·43	2·35

Table 5 shows the effects of nicotinamide and homocystine, and the combined effects of nicotinamide and creatinine and nicotinamide and acetone. Nicotinamide (0·1%, w/v) and homocystine (0·1%, w/v) depressed choline biosynthesis. This depression was, however, completely or partially counteracted if creatinine or acetone was applied simultaneously. Creatinine (0·1%, w/v) reduced the depressant effect of nicotinamide (0·1%, w/v) from 29·2 to 15·2%, and acetone (1%, v/v) completely abolished it.

DISCUSSION

The results (Table 1) indicating that the choline content of the seedling increased gradually during the first 96 hr. and then declined and that more of the choline accumulated in the growing part of the embryo (shoot plus radicle) with a concomitant decrease in the cotyledons as germination progressed may be regarded as indications that choline biosynthesis bears a direct relationship to the metabolic activity of the growing seedling. One may suspect that after 96 hr. one or more of the nutrients required by the growing seedling became limiting, resulting in a subsequent decline in the choline content of the seedling. The maximum amount of choline was found to have been synthesized during the first 96 hr. irrespective of the addition of potassium, ammonium, or nitrate ions (Table 2), or of methionine, creatinine, acetone or methanol (Table 4), or of thiamine or riboflavin (Table 3). It is difficult to conclude from the determination of only one product, namely choline, whether the added supply of these substances accelerated the overall process of germination in the seedling. The seeds appeared to germinate at about the same time in all cases after treatment with the germinating fluid. Potassium is a well-known activator of many enzyme systems and probably stimulated choline biosynthesis by enhancing some enzyme processes concerned in choline biosynthesis, which needed more potassium for their maximum activity. Ammonium and nitrate ions may increase the nitrogen metabolism of the seedling in favour of choline biosynthesis only by providing additional nitrogen.

The effect of riboflavin and thiamine may be traced to their role as constituents of many enzyme systems. It is not known, however, if these vitamins have similar effects on choline biosynthesis in the animal. Unlike the B vitamins, ascorbic acid is not present in the seed but is produced during germination and may change the oxidation-reduction potential of the germinating system (Williams, Eakin, Beerstecher & Shive, 1950), which may cause a depression of choline biosynthesis.

Methionine, acetone and methanol are known to give rise to choline methyl groups in the animal, although quantitative data regarding their relative potency are not available. Du Vigneaud, Chandler, Cohn & Brown (1940) demonstrated the transfer of the methyl group of methionine to choline in the rat, the reverse process, i.e. the formation of methionine from homocystine and choline having been established earlier (Du Vigneaud, Chandler, Moyer & Keppel, 1939). Sakami (1950) noted that ^{14}C-labelled acetone gives rise to the methyl carbon of choline in the rat, and it has been shown that this transformation may occur via formate (Welch & Sakami, 1950). Du Vigneaud & Verly (1950) found that methanol may serve as precursor of choline methyl groups and Du Vigneaud, Verly & Wilson (1950) demonstrated the formation of choline methyl groups from formate and formaldehyde in the rat. The contribution of formate and methionine to the methyl group of nicotine in *N. rustica* L., and of hordenine, creatine and choline in wheat seedlings has been mentioned earlier. Though glycocyamine is methylated by rat liver in the presence of methionine to give creatine (Borsook & Dubnoff, 1945), the reverse process, i.e. the formation of methionine from homocystine and creatine, could not be demonstrated (Du Vigneaud, Chandler & Moyer, 1941). The experiments presented in this paper show that DL-methionine, creatinine, acetone and methanol cause stimulation of choline biosynthesis in the seedlings of *C. arietinum* presumably by contributing to the synthesis of methyl groups. It is also shown (Table 4) that DL-methionine is better than creatinine, creatinine is better than acetone and acetone is better than methanol as a source of methyl groups.

Nicotinamide (Handler & Dunn, 1942) and homocystine (Du Vigneaud *et al.* 1940) are well-known acceptors of methyl groups in the animal. These compounds may therefore depress choline biosynthesis in the seedlings of *C. arietinum* by competing for methyl groups (Table 5). By providing additional methyl groups, creatinine and acetone reduce or eliminate this effect of nicotinamide when either of them is present together with nicotinamide during germination. The observation that acetone is more effective than creatinine in counteracting the depressant effect of nicotinamide, though the latter stimulated choline biosynthesis to a greater extent when applied alone, will need further exploration.

SUMMARY

1. The biosynthesis of choline in the germinating seedling of the chick-pea (*C. arietinum*) has been studied.

2. During the first 96 hr. the choline content of the seedling increases to a maximum value of 3·78 mg. choline chloride/g. of seedling and then gradually declines.

3. Potassium, ammonium and nitrate ions stimulate choline biosynthesis. A similar effect is produced by thiamine and riboflavin while ascorbic acid depresses choline synthesis.

4. DL-Methionine, creatinine, acetone and methanol stimulate the biosynthesis of choline by 31·6, 29·2, 9·2 and 4·2%, respectively, after 96 hr. of germination.

5. Nicotinamide and homocystine depress choline biosynthesis in the seedling. This depression is however wholly or partially counteracted by creatinine or acetone.

REFERENCES

Barrenscheen, H. K. & Pany, J. (1941). *Biochem. Z.* **310**, 344.

Barrenscheen, H. K. & Valyi-Nagy, T. von (1942). *Hoppe-Seyl. Z.* **277**, 97–113.

Borsook, H. & Dubnoff, J. (1945). *J. biol. Chem.* **160**, 635.

Brown, S. A. & Byerrum, R. U. (1952). *J. Amer. chem. Soc.* **74**, 1523.

Du Vigneaud, V., Chandler, J. P., Cohn, M. & Brown, G. B. (1940). *J. biol. Chem.* **134**, 787.

Du Vigneaud, V., Chandler, J. P. & Moyer, A. W. (1941). *J. biol. Chem.* **139**, 917.

Du Vigneaud, V., Chandler, J. P., Moyer, A. W. & Keppel, D. M. (1939). *J. biol. Chem.* **131**, 57.

Du Vigneaud, V. & Verly, W. G. (1950). *J. Amer. chem. Soc.* **72**, 1049.

Du Vigneaud, V., Verly, W. G. & Wilson, J. E. (1950). *J. Amer. chem. Soc.* **72**, 2819.

Handler, P. & Dunn, W. J. (1942). *J. biol. Chem.* **146**, 357.

Kirkwood, S. & Marion, L. (1951). *Canad. J. Chem.* **29**, 30.

Luecke, R. W. & Pearson, P. B. (1944). *J. biol. Chem.* **155**, 508.

McIntire, J. M., Sweigert, B. S. & Elvehjem, C. A. (1944). *J. Nutr.* **28**, 219.

Sakami, W. (1950). *Fed. Proc.* **9**, 222.

Steensholt, G. (1946). *Acta Physiol. scand.* **11**, 136–40.

Welch, A. D. & Sakami, W. (1950). *Fed. Proc.* **9**, 245.

Williams, R. J., Eakin, R. E., Beerstecher, E. jun. & Shive, W. (1950). *The Biochemistry of B vitamins*, Amer. chem. Soc. Monograph no. 110, p. 21. London: Chapman; New York: Reinhold.