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Role of Carbon Dioxide in Citric Acid Fermentation: Part I — Incorporation of Carbon Dioxide into Citrate*†

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Tracer studies with $C^{14}O_2$ have indicated that carbon dioxide in the atmosphere above the fermenting surface cultures of *Aspergillus niger* plays an important role in the synthesis of citric acid. It has been demonstrated that under suitable conditions up to 60 per cent of atmospheric $C^{14}O_2$ is incorporated into citric acid and as much as one-sixth of the total carbon in the citrate molecule is derived entirely from carbon dioxide. The rate of incorporation of carbon dioxide into citrate is a non-linear process varying with the age and the nature of the chemical activity of the mould. The implications of these findings in the light of accepted mechanisms of citrate biogenesis are discussed.

FOSTER *et al.*[1] were the first to demonstrate with the aid of isotopic tracer technique that carbon dioxide was incorporated into citrate by *Aspergillus niger*. The initial discovery of Foster was followed up by a number of detailed investigations by different workers on various aspects of carbon dioxide incorporation into citrate[2-10]. These studies have contributed towards a better understanding of the mechanisms involved in the synthesis of citric acid in *A. niger* and other biological systems. In most of these investigations preformed mycelia from either surface or submerged cultures of the mould were employed. Under these conditions good yields of citric acid were not obtainable in most cases. Therefore, the proportion of citrate carbons[4] estimated to be derived from carbon dioxide is likely to be lower in these experiments than that actually obtainable under more favourable conditions for citric acid fermentation. Even in the more recent work of Cleland and Johnson[10], where the amount of citric acid formed accounted completely for the utilized sugar, the inoculum was rather large and the exact extent of participation of carbon dioxide in citrate synthesis was probably obscured due to the presence of a large pool of endogenous unlabelled metabolites compared to that of labelled $C^{14}O_2$. Hence the percentage of citrate carbons derived from CO_2 (calculated from Cleland and Johnson's data) did not exceed 7·3 per cent.

In order to assess the quantitative role of carbon dioxide citric acid fermentation, it was necessary to study the incorporation of $C^{14}O_2$ in citrate, as far as practicable, under conditions of maximum citric acid formation by the mould. The present paper describes the results obtained under such conditions with surface cultures of *A. niger*.

Experimental procedure

The mould — The culture of *A. niger* used was the A_5 strain which has been used in earlier studies in this laboratory[11,12]. Dry spores from a seven days old slant culture on potato-dextrose agar were used for inoculation.

Media — The basal medium used was modified Currie's medium[11,12] with glucose or cane sugar as the carbon source. The sucrose media were supplemented with two levels of zinc (114 and 171 μg. Zn^{2+}/l) as $ZnSO_4.7H_2O$. Glass-distilled water was used in the preparation of media and solutions throughout the experiment.

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Estimations — (i) Residual sugar was estimated by the anthrone method[13]; (ii) acidity was determined by titration against 0·1*N* sodium hydroxide with phenolphthalein as indicator; (iii) citric acid was estimated according to Natelson *et al.*[14] and (iv) the liquid and the solid samples were subjected to wet and dry combustions respectively according to Bhattacharyya[15] and the radioactivity measurements were made according to Das Gupta and Nair[16].

Apparatus and procedure — Preliminary studies[12] on the oxygen requirement for citric acid fermentation in stationary surface cultures indicated that the final yield and the rate of citric acid formation were unaffected in airtight enclosed systems provided the volume of enclosed air was not less than 250 ml. per millimole of hexose fermented. These studies were useful in the design of the equipment and techniques for subsequent tracer studies with $C^{14}O_2$.

The Pyrex all-glass apparatus used for studying the incorporation of carbon dioxide is illustrated in Fig. 1. In the culture tube 'A' sterile medium (1·5 ml. containing 180 mg. of sugar) was seeded with *A. niger*. $C^{14}O_2$ was generated in the enclosed system

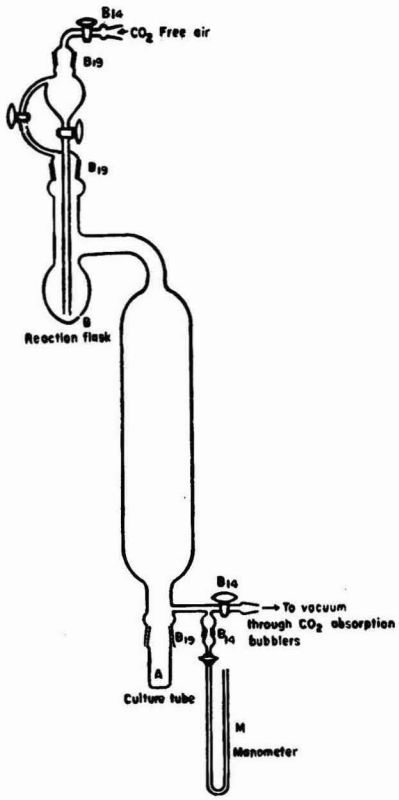


Fig. 1 — Apparatus for the fixation of carbon dioxide

at 28°C. under slightly reduced pressure by dropping two volumes of 50 per cent sulphuric acid from the pressure-equalizing dropping funnel to one volume of Na_2CO_3 solution in the CO_2-generating flask (B). The stopcock leading in CO_2-free air was opened and the pressure inside the system was brought to the normal atmospheric level by bubbling CO_2-free air through the liquid in (B). The system was then completely closed to the atmosphere. Two such assemblies were placed in a large incubator maintained at 28° ± 0·5°C. and the fermentation was allowed to proceed for 7-9 days. Nine control tubes, identical in shape and size with the experimental culture tube A, were also inoculated and incubated simultaneously enclosed in glass-stoppered iodine flasks having the same capacity as that of the CO_2 fixation apparatus (*c.* 500 ml.). These were withdrawn in duplicates at suitable intervals (daily from the fifth day onwards) and their contents titrated to determine the progress of acid production. The minimum pressure registered in the manometer (M) with the apparatus also coincided with peak of acid production (oxygen depletion from the enclosed system caused this depression).

With glucose as substrate the maximum yield of citric acid was obtained in 8-10 days, whereas on a cane sugar medium supplemented with zinc, maximum acidity was obtained in 7 days.

In all these experiments citric acid accounted for all the titrable acidity of the fermented medium. At the end of the fermentation the system was evacuated by an aspirator pump through two CO_2 absorption bubblers connected in series, each containing 20 ml. of CO_2-free 1*N* sodium hydroxide and the residual carbon dioxide was swept out by CO_2-free air let in in the form of an air-leak through the CO_2 generator B. Total respiratory CO_2 and its specific activity were estimated[16].

The contents of the culture tube A were filtered. The mycelium was washed repeatedly with CO_2-free water, pressed as dry as possible with a glass rod, dried over phosphorus pentoxide *in vacuo* and estimated for radioactivity. The filtrate and mycelial washings were made up to 10 ml. with CO_2-free water (solution A).

An aliquot of 1 ml. of solution A was titrated for acidity and the neutral solution obtained after titration was made up to 10 ml. with CO_2-free water (solution B).

Aliquots from solution B were used for determination of residual sugar, citric acid and radioactivity.

Several experiments were run with either glucose or cane sugar as substrates. The carbon and radioactivity balances of six such experiments are summarized in Table 1.

TABLE 1 — CARBON AND RADIOACTIVITY BALANCE

Exp. No.:	G_1		G_2		G_3*		CS_1†		CS_2†		CS_3‡	
Yield§, % :	35·6		72·2		52·2		70·3		45·3		57·5	
Conversion yield¶, % :	44·8		75·4		69·0		79·1		53·0		58·1	
	C	A	C	A	C	A	C	A	C	A	C	A
Respiratory CO_2	20·6	35·5	20·1**	33·75	28·0	59·6	15·2**	41·7**	32·0	77·2	36·0	64·6
Mycelium	16·7	17·0	18·1	6·75	22·3	4·5	17·6	1·9	19·9	1·8	15·3	1·2
Medium	—	35·5	—	59·5	—	35·4	—	56·4	—	25·6	—	37·8
Residual sugar	15·5	—	Negligible	—	11·0	—	10·4	—	10·8	—	4·5	—
Citric acid	30·4	—	61·8	—	46·0	—	56·8	—	36·7	—	46·6	—
Total	83·2	88·0	100·0	100·0	107·3	99·5	100·0	100·0	99·4	104·6	102·4	103·6

C and A denote percentages of initial carbon and initial radioactivity.
*With 57 μg. Zn^{2+}/litre.
†With 114 μg. Zn^{2+}/litre.
‡With 171 μg. Zn^{2+}/litre.
§Yield = g. of citric acid monohydrate/100 g. of available sugar.
¶Conversion yield = mole of citric acid/100 mole of (utilized sugar — sugar equivalent of mycelial carbon).
**Estimated by difference only.

TABLE 2 — SPECIFIC ACTIVITIES OF RESPIRATORY CO_2, MYCELIAL CARBON, CITRIC ACID AND CARBOXYLS OF CITRIC ACID

Exp. No.:	G_1	G_2	G_3	CS_1	CS_2	CS_3
Initial CO_2, μc.	40·00	40·00	15·70	15·80	15·80	15·80
Final CO_2, μc./μmoles × 10^3	11·50	11·04	5·33	6·26	6·02	4·47
Mycelium, μc./μmoles × 10^3	6·70	2·49	0·46	0·27	0·22	0·19
Citric acid, μc./μmoles × 10^3	43·10	38·30	13·00	15·27	10·42	12·72
*Primary CO_2H, μc./μmoles × 10^3	26·00	22·10	6·74	8·62	6·24	8·08
Tertiary CO_2H, μc./μmoles × 10^3	16·70	13·70	6·18	6·63	4·01	4·69
Ratio, $y = \frac{1°CO_2H}{3°CO_2H}$	1·55	1·62	1·09	1·30	1·55	1·72
K	11·56	9·90	11·60	7·86	11·25	15·55
†Theoretical values of K, K_0	22·05	21·81	24·32	23·07	22·05	21·51
†Efficiency of CO_2 incorporated $100K/K_0$ (%)	52·40	45·40	47·70	34·10	51·00	72·10

*Assuming that one of the two primary carboxyls is labelled.
†For explanation see discussion of results.

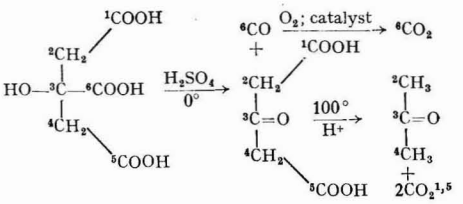
In Exp. G_1 (Table 1), after the addition of 150 mg. of inactive carrier citric acid, solution A was passed through a column of Amberlite IRA-45 in the hydroxyl phase to remove the acidic constituents of the medium. The effluents from the column containing all the unfermented sugar were found to be devoid of any significant radioactivity.

Citric acid, accounting for practically all the radioactivity of the medium, was eluted from the column with 1*N* ammonium hydroxide and isolated as the sparingly soluble quinidine salt.

In all other experiments reported in Table 2 citric acid was directly isolated from solution A as quinidine citrate which was purified by repeated recrystallizations from hot water either to a constant specific activity or to a constant solubility. The melting point (134-6°C.) did not always indicate the purity of the compound. Estimations of citric acid were carried out on the recrystallized samples to confirm their purity.

The specific activities of quinidine citrate samples were determined on 3-8 mg. samples by the dry oxidation method[15].

Degradation of citric acid — Aliquots of 10-15 mg. of the samples of quinidine citrate were degraded with conc. H_2SO_4 essentially according to the method of Lewis *et al.*[5]. The sequence of reactions is given below:



A few modifications were introduced in the conventional set-up for carrying out this degradation. The apparatus is illustrated in Fig. 2. The degradation of citrate was carried out as follows.

The sample of quinidine citrate was placed in the reaction flask (A) which was connected to the assembly in the manner indicated in the diagram and surrounded by an ice-salt bath (−10° to −5°C.). The apparatus was continuously flushed with CO_2-free oxygen with the help of suction applied at the end of bubbler

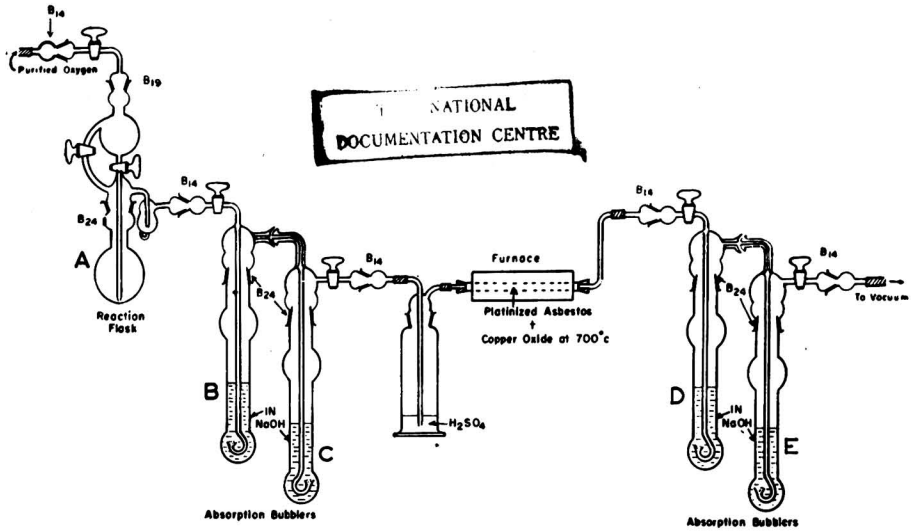


Fig. 2 — Assembly for the degradation of citrate

E. The copper catalyst was heated to 400°C. and maintained at this temperature throughout the experiment. At the end of 1 hr (the time needed for the activation of the catalyst) ice-cold concentrated sulphuric acid (2 ml.) was poured into the reaction flask (A) through the pressure-equalizing dropping funnel and the decarbonylation reaction allowed to proceed in the cold for 1 hr. The ice-salt bath was then replaced with a boiling water bath and the flask (A) was heated for 2 hr for the completion of the decarboxylation reaction.

Carbon dioxide liberated from the primary carboxyls was trapped in the first two bubblers, (B) and (C). The carbon monoxide from the tertiary carboxyl was oxidized to carbon dioxide and was absorbed in bubblers (D) and (E).

It was found that the carboxyl carbons accounted for all the radioactivity incorporated into citrate. In experiment G_1 the acetone from citrate degradation (carbons marked 2, 3 and 4) was isolated as Denige's salt[6] and found to contain no significant radioactivity above that of the background.

Citric acid carbons derived from CO_2 — The percentage of citrate carbons derived from CO_2 was calculated according to Martin and Wilson[4,8] assuming a linear evolution of carbon dioxide and a linear production of citric acid throughout the experimental period. It is necessary to summarize the calculations here in an algebraic form:

When initial $CO_2 = a$ μmoles
and final $CO_2 = b$ μmoles, then

the dilution factor, $f = \frac{a+b}{2a}$ . . . . . (1)

If r μcuries and c μcuries be the initial radioactivity in CO_2 and the final radioactivity in citrate respectively, the fraction of radioactivity in citrate,

$$x = \frac{c}{r} \quad \ldots\ldots\ldots\ldots \quad (2)$$

Then the CO_2 pick-up in citrate, $p = afx$ μmoles, or substituting from equations (1) and (2)

$$p = \frac{ac(a+b)}{2ar} \text{ μmoles} \quad \ldots\ldots\ldots \quad (3)$$

If the total citrate carbons $= q$ μmoles, then the percentage of citrate carbons derived from CO_2

$$K = \frac{p}{q} \times 100 = \frac{100ac(a+b)}{raq} = \frac{50c(a+b)}{rq} \quad \ldots \quad (4)$$

The specific activities of carboxyl carbons and different fractions from the six experiments and the K values are reported in Table 2.

CO_2 incorporation at different stages of citric acid fermentation — In some experiments described so far, as much as 55-60 per cent of the initial atmospheric radioactivity were found to be incorporated into citrate (Table 1, Exp. G_2, CS_1). The question whether the CO_2 was incorporated at a uniform rate throughout the experimental time of nine days (with glucose) had to be settled. The experimental time was divided into three different periods based on the chemical activity of the mould, viz. (i) between 0 and the fifth day when the mycelium was formed; (ii) between the fifth and the seventh day when the rate of citric acid synthesis usually reached a maximum and (iii) between the seventh and the ninth day when the accumulation of citrate levelled off.

TABLE 3 — CARBON AND RADIOACTIVITY BALANCE OF EXPERIMENTS AT DIFFERENT AGES OF THE MOULD

[*Carbon (C) expressed in μmoles and radioactivity (A) in μcuries; total initial carbon from glucose, 6000 μmoles and from CO_2, 18 μmoles; total, 6018 μmoles*]

Incubation period *days*	Tube No.	Mycelium		Respiratory CO_2		Medium		Total			Recovery, %	
		C	A	C	A	Glucose	A	Citrate	C	A	C	A
0-5	1	275* (275)	0·46	776 (758)	14·97†	4367	0·37	600 (600)	6018	15·80	100·0	100·0
	2	450 (450)	2·46	577 (559)	10·90	4660	0·34	240 (240)	5927	13·70	98·6	90·2
5-7	3	900 (475)	0·13	648 (73)	12·50†	2550	3·17	1920 (1143)	6018	15·80	100·0	100·0
	4	848 (428)	0·12	1279 (699)	12·39	2667	2·33	1500 (714)	6291	14·86	103·5	94·0
7-9	5	1172 (298)	0·11	2176 (900)	14·12	821	2·60	2836 (829)	7005	16·83	116·0	106·5
	6	1568 (694)	0·05	2219 (943)	14·20	—	2·63	3570 (−11)	7347	16·88	122·0	106·8

*The figures in parentheses represent the increment within the experimental period.
†Calculated by difference.

Six culture tubes were inoculated and two of them were placed in the two CO_2 fixation assemblies (Fig. 1). $C^{14}O_2$ (15·8 μcuries, 10 μmoles) was generated in the enclosed system in the usual manner. The remaining four tubes were incubated in separate enclosures. After five days the respired carbon dioxide was collected from both the assemblies and estimated. The five days' old cultures were removed for analysis. The assemblies were dismantled, washed, dried and set up again. Two fresh five days' old cultures were removed from the enclosures. The medium below the mycelial mats in these tubes was carefully mixed by withdrawing and expelling the liquid by a thin-tipped Pasteur pipette without disturbing the mat. Small aliquots (0·05 ml.) were removed for analysis by a drawn out capillary pipette. Both these tubes were then replaced in the assembly. Radioactive $Na_2C^{14}O_3$ (15·8 μ) and enough carrier sodium carbonate calculated to yield the average amount of respired carbon dioxide found in the assemblies after the five-day experiment were placed in the CO_2 generating flask (B) and acid in the dropping funnel. The systems were partially evacuated and $C^{14}O_2$ was generated in the enclosed system. The pressure was brought back to the atmospheric level by allowing CO_2-free air to bubble through the flask (B). The systems were then closed again and the fermentation was allowed to continue up to the seventh day.

The whole cycle of operations was repeated again on the seventh day and the third stage of fermentation carried out from the seventh to the ninth day by replacing the culture tubes (A) with two more seven-day cultures grown in an enclosed system.

TABLE 4 — SPECIFIC ACTIVITIES OF DIFFERENT FRACTIONS FROM EXPERIMENTS RUN AT DIFFERENT AGES OF THE MOULD

Incubation period *days*	Tube No.	Respiratory CO_2 *μc./μmole* $\times 10^3$	Mycelial carbon *μc./μmole* $\times 10^3$	Citric acid* *μc./μmole* $\times 10^3$	*K* %
0-5	1	—	1·27	3·6	1·6
	2	21·10	5·47	8·5	2·4
5-7	3	—	2·65	13·0†	10·8
	4	9·70	0·29	19·6†	16·6
7-9	5	6·49	0·37	18·8†	27·8
	6	6·40	0·08	‡	‡

*Calculated on the increment of citric acid values within parentheses in Table 3.
†Sample errors of ±5 per cent would cause a variation of ±20 per cent in extreme cases.
‡Citric acid decreased from the seventh to the ninth day.

Table 3 illustrates the radioactivity and carbon balance of this experiment (the carbon balance figures were calculated for tubes 3-6 on the assumption that the two stages for each tube add up to a single uninterrupted experiment for seven or nine days).

In Table 4 are given the specific activities of different fractions and the percentage of citrate carbons (*K*) derived from CO_2.

Discussion

It is pertinent to discuss the results of these experiments in the light of the known mechanisms for citrate synthesis.

Mechanism of incorporation of carbon dioxide in a primary carboxyl group of citrate — In experiments

using either 3,4-labelled glucose or $C^{14}O_2$ Cleland and Johnson[10] surmised that a C_3 and C_1 condensation followed by a C_4 and C_2 condensation appears to be an important mechanism for citrate synthesis. Our data are not inconsistent with this interpretation.

Four enzyme systems have been implicated in the C_3-C_1 condensation process in *A. niger*. Of these no net synthesis of oxalacetate can occur with β-carboxylase[17] since equilibrium conditions are unfavourable. The evidence regarding the existence of malic enzyme[18,19] in *A. niger* is controversial[20]. The equilibria in two other systems, viz. phosphopyruvic carboxykinase and another enzyme which synthesizes oxalacetate from pyruvate and CO_2 through the mediation of ATP[20], are not unfavourable for a net synthesis of oxalacetate. The condensing enzyme which can bring about the C_2-C_4 condensation is known to be present in *A. niger*[21].

The origin of labelling in the tertiary carboxyl group —In all the experiments reported here, both the primary and the tertiary carboxyl groups incorporated $C^{14}O_2$ and the ratio of specific activities of these two carbons (Y) was between 1·1 and 1·7 (1·7, Table 2). According to the Ogston concept[22] or the more general 'meso' carbon concept[23,24] it may be assumed that only one of the two primary carboxyls of citrate — that derived from oxalacetate — would be labelled.

The appearance of radioactivity in the middle carboxyl of citrate was also observed in all other previous investigations and was explained as being due to an extensive and fast equilibration among the four carbon dicarboxylic acids of the TCA cycle in the following manner:

Oxalacetate-4-C^{14} ⇌ malate-4-C^{14} ⇌ fumarate-4-C^{14}
↓ ⇅
citrate-1-C^{14} fumarate-1-C^{14}
⇅
malate-1-C^{14}
⇅
oxalacetate-1-C^{14}
↓
citrate-6-C^{14}

It was assumed that since fumarate is symmetrical it was no longer possible to distinguish between the two carboxyls. However, from the 'meso' carbon concept[24] the fumarate molecule was predicted to behave in a stereospecific manner in enzyme systems. More recent studies have fully justified such a prediction[25]. Therefore, the carboxyl groups of fumarate can no longer be considered to be equivalent.

The only member of the TCA cycle which behaves in a completely non-stereospecific manner is succinate[26]. So, in order to explain the appearance of radioactivity in the tertiary carboxyl group of citrate, one has to consider whether oxalacetate can go three steps to succinate and back again before being condensed into citrate. This hypothesis runs into some difficulties as it is known that the reversal of succinic dehydrogenase system can only be demonstrated under anaerobic conditions[27]. At the same time it is also difficult to explain the randomization of labelling in the citrate carboxyls through alternative pathways leading to succinate.

The TCA cycle cannot be considered as an alternative mechanism of succinate formation since the aconitase system is either absent[21] or inoperative under the conditions of citric acid fermentation.

A combination of malate synthetase[28-30] and the succinate cleaving enzyme[31,32] acting in the reverse direction to provide a route from malate to succinate is not feasible, since malate synthetase is not known to be reversible[33].

A fourth mechanism implicated in the synthesis of succinate involving propionyl-CoA carboxylase[34], methyl malonyl-CoA isomerase[35], transcarboxylase[36] and deacylase[37] also appears to be untenable since it is difficult to account for the formation of stoichiometric amounts of propionyl-CoA.

Alternative pathways and the significance of CO_2 incorporation into citrate: *A net fixation or exchange* — Foster and Carson[3] pointed out the possibility of a C_2-C_2 type condensation in moulds leading to a C_4-dicarboxylic acid. Assuming that a convenient enzymic mechanism also exists in *A. niger* for this type of condensation, two broad overall pathways emerge by which labelled carbon dioxide from the atmosphere may appear in the primary and the tertiary carboxyl carbons of citrate. These are schematically represented in Charts I and II.

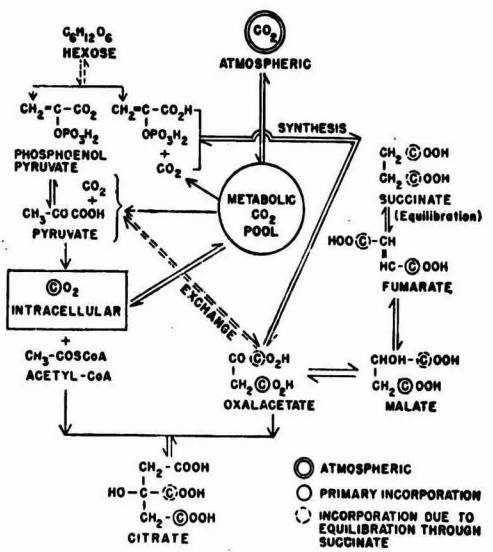


Chart I — Pathway No. 1 for the incorporation of carbon dioxide in a primary carboxyl group of citrate involving no loss of hexose carbons as carbon dioxide

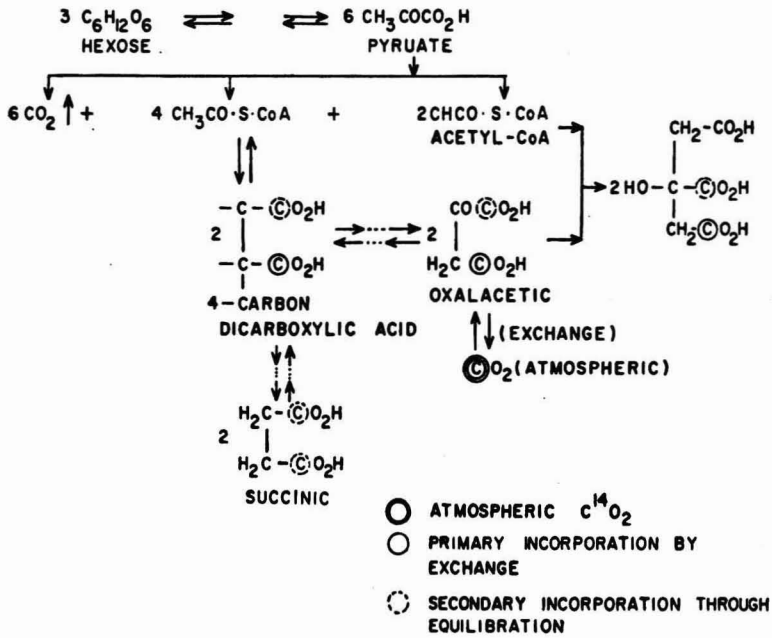


Chart II — Pathway No. 2 for the incorporation of carbon dioxide in a primary carboxyl group of citrate involving 33 per cent loss of hexose carbons as carbon dioxide

The distinctive features of pathway No. 1 (Chart I) are as follows:

(i) Carbon dioxide is incorporated into citrate by a net fixation or synthesis. A hexose molecule is broken down to two molecules of phosphoenolpyruvate. One molecule proceeds to pyruvate and liberates acetyl-coenzyme A and carbon dioxide. This carbon dioxide goes into the metabolic pool, picks up labelling by equilibration with the incoming $C^{14}O_2$ from the atmosphere and is condensed with the remaining molecule of phosphoenolpyruvate to form carboxy-labelled oxalacetate which can give rise to labelled citrate.

(ii) All the six carbons of the hexose molecule eventually appear in citrate. The maximum possible yield of citrate by this pathway would be 100 per cent on a molar basis.

In pathway No. 2 (Chart II) no direct incorporation of CO_2 by a net synthesis is possible. Pyruvic acid is oxidatively decarboxylated to a two-carbon unit and carbon dioxide. Two two-carbon units are presumed to condense to a four-carbon dicarboxylic acid which condenses with another two-carbon unit to form citrate at the oxalacetate stage. The maximum possible molar yield of citrate can never exceed 66·7 per cent by this pathway since two hexose carbons are lost as carbon dioxide.

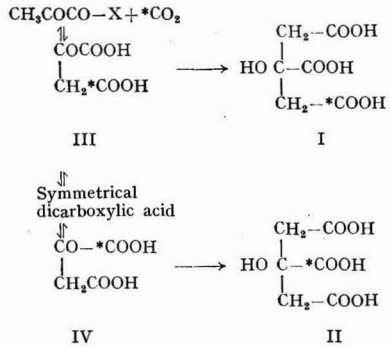
It is possible, however, to obtain labelled citrate by this pathway by postulating an exchange of carboxyl carbons at the oxalacetate or malate level. In fact, this possibility is common to both the pathways.

Earlier investigators in this field could not come to a definite conclusion as to whether the CO_2 incorporation in citrate is due to a net fixation or an exchange. Nor do the isotopic data obtained in the current investigation permit a differentiation between these two modes of CO_2 incorporation. It is interesting to note, however, that in all the experiments reported in Table 2 the specific activities of the primary carboxyls of citrate (*l.* 5) were higher than the final specific activities of respired carbon dioxide (*l.* 2). This does not necessarily mean that a net synthesis had taken place. A higher specific activity of the carboxyl carbons may also be explained by assuming the release of citric acid into the medium to be an irreversible transport process, irrespective of whether the radioactivity of the carboxyl carbons were derived by a net synthesis or an exchange.

Fortunately, as was pointed out by Wells *et al.*[37], a judgement between the possible pathways is feasible on the criterion of yields. In two of the experiments recorded in Table 1 (G_2 and CS_1) conversion yields of the order of 76 and 79 per cent were obtained. At least in these experiments a considerable amount of citric acid was synthesized by pathway No. 1 through a net fixation of carbon dioxide. Occasionally in control tubes run along with tracer experiments, conversion yields of the order of 97-99 per cent have been achieved. In those tubes

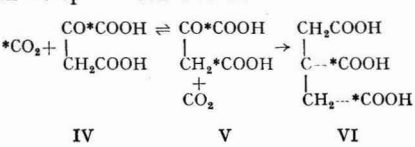
pathway No. 1 was the only significant route to citrate synthesis. In the experiments in which the conversion yields were less than 67 per cent, no choice is possible between the two pathways.

Percentage of citrate carbons derived from carbon dioxide — Martin and Wilson[4,8], who calculated the percentage of citrate carbons derived from carbon dioxide, had assumed that only two species of labelled citrate, one labelled in one of the primary carboxyls (I) and the other in the tertiary carboxyl (II), may arise by isotopic carbon dioxide incorporation through the oxalacetates (III) and (IV) respectively.



On this basis it was presumed that the maximum percentage of citrate carbons derivable from CO_2 would be 16·7 per cent (one carbon out of six).

However, there is a third possibility which seems to have been overlooked by earlier investigators. A third species of citrate (VI) in which both the carboxyls are labelled may arise through an exchange of the β-carboxyl carbon of oxalacetate species IV, with isotopic carbon dioxide.



However, this exchange with oxalacetate species III would lead to singly labelled citrate.

This would require a revision of the theoretical maximum value for K (K_0) to $16{\cdot}7\left(1+\frac{1}{2y}\right)$ where y = ratio of specific activities of two carboxyls. The last two lines in Table 2 give the calculated values of K_0 and the efficiency of incorporation.

It may be observed from Table 2 that the efficiency of incorporation in the first five experiments was between 35 and 52 per cent. Exp. CS_3 was rather anomalous, where both K and the efficiency of incorporation were abnormally high, presumably due to some breakdown of citrate (*vide infra*). It is also

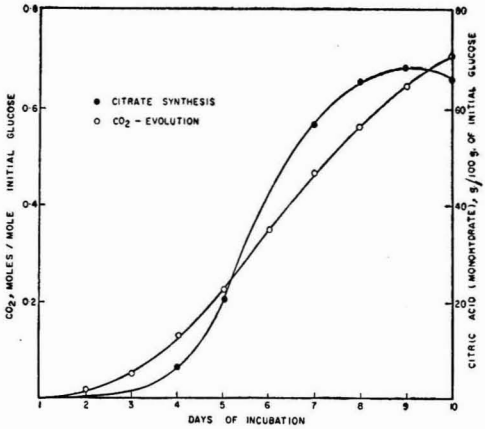


Fig. 3 — Rates of carbon dioxide evolution and citrate synthesis

obvious that the ratio y, the values of K and the efficiency of incorporation were totally unrelated to the final yields of citric acid. This lack of correlation between values of K and the citric acid yields is suggestive of the existence of an exchange incorporation.

The rates of citrate synthesis and CO_2 incorporation — In the calculation of values for K in equation (4) it was assumed that both the CO_2 evolution and citrate synthesis were linear processes throughout the experimental period. From Fig. 3, which gives the rates of CO_2 evolution and citrate synthesis, it is obvious that these assumptions were not strictly valid and the deviation from linearity was quite appreciable at the earliest and the last phase of the fermentation. It was, therefore, decided to divide the fermentation into three stages: (1) preliminary (0-5th day), (2) intermediate (5th-7th day) and (3) final (7th-9th day).

(1) The first stage was characterized by a rapid rate of mycelium formation and a logarithmic rate of CO_2 evolution. Only small amounts of citric acid were formed. The values of K (Table 4) were low at this stage presumably due to any or all of the following reasons: (i) lower partial pressure of isotopic CO_2, (ii) a higher rate of CO_2 evolution due to presence of other mechanisms at the initial state (HMP pathway, TCA cycle, etc.) and (iii) inaccuracies in calculation due to deviation from linearity.

(2) During the intermediate phase the maximum rates of citrate synthesis were attained. The value of K reached up to a level of 16·6 per cent in tube No. 4 (approximately one carbon out of six). The deviation from linearity at this stage was minimum for both the processes — CO_2 evolution and citrate synthesis.

(3) During the final phase the values for K were found to rise still further (Table 4; tube No. 5). However, at this stage, due to a depletion of carbohydrate source, the mould was likely to metabolize some citrate for growth*. This could increase the apparent value of K abnormally.

During the final phase one can assume that two processes — anabolism and catabolism of citrate — were operating simultaneously. Isotopic carbon dioxide was participating in the former process giving rise to labelled citrate. The citrate formed was being diluted with unlabelled preformed citric acid in the medium for the catabolic process.

Under such conditions in equation (4) the value of c would be higher and that of q lower, increasing the value of K abnormally. The implication of this would be clear from the data obtained for tube No. 6 (Tables 3 and 4) where a fair amount of $C^{14}O_2$ was incorporated, although the yield of citric acid went down from the seventh to the ninth day.

Finally, it is interesting to note that in Exp. G_1 (Table 1), the residual glucose separated from citric acid by an ion-exchange column was found to be devoid of radioactivity. By analogy with other animal systems it is expected that the mould polysaccharides (starch or glycogen) and hence the intracellular glucose should pick up some labelling from atmospheric $C^{14}O_2$ by a reversal of glycolysis. The amounts of mycelia isolated in these experiments were far too small to investigate this possibility. However, it appears probable that under the experimental conditions there is no exchange between intracellular and extracellular glucose.

In conclusion, it can be stated that in spite of a considerable amount of work done during the past twenty years with isotopic tracers and enzymes, the precise mechanism of citrate synthesis in *A. niger* remains to be rigorously established. The accumulation of citrate in the medium may be a ' cybernetically ' regulated process influenced by multiple ' feedback control ' mechanisms. The mere demonstration that a particular enzyme is present does not necessarily prove that it is involved or active during the synthesis of citrate. For instance, the ' Zwischenferment ' is known to occur in *A. niger* in considerable amounts even under the optimum conditions for citric acid production[38]. However, the hexose monophosphate pathway in which as many as three carbons of glucose may be lost as carbon dioxide is obviously not operative under these conditions as often all the six carbons of hexose are quantitatively accounted for in the six carbons of citrate.

*In control experiments run with 50 μmoles of radioactive citrate isolated from Exp. G_2 along with glucose it was found that no appreciable radioactivity could be detected in the atmosphere due to degradation of citrate up to the eighth day. However, on the ninth day traces of radioactive $C^{14}O_2$ appeared in the atmosphere.

Acknowledgement

The authors would like to express their thanks to Dr V. Jagannathan for his interest in this work.

References

1. Foster, J. W., Carson, S. F., Ruben, S. & Kamen, M. D., *Proc. nat. Acad. Sci., Wash.*, **27** (1941), 590.
2. El-Kerdany, M. H., *Fixation of Heavy Carbon Dioxide by Aspergillus niger*, Master's Thesis, Iowa State College, 1947.
3. Foster, J. W. & Carson, S. F., *J. Amer. chem. Soc.*, **72** (1950), 1865.
4. Martin, S. M., Wilson, P. W. & Burris, R. H., *Arch. Biochem.*, **26** (1950), 103.
5. Sprites & Scelles, cf. Stern, J. R., Shapiro, B. & Ochoa, S., *Nature, Lond.*, **166** (1950), 403.
6. Lewis, K. F. & Weinhouse, S., *J. Amer. chem. Soc.*, **73** (1951), 2500, 2906.
7. Carson, S. F., Mosbach, E. M. & Phares, E. F., *J. Bact.*, **62** (1951), 235.
8. Martin, S. M. & Wilson, P. W., *Arch. Biochem. Biophys.*, **32** (1951), 150.
9. Mosbach, E. M., Phares, E. F. & Carson, S. F., *Arch. Biochem. Biophys.*, **35** (1952), 435.
10. Cleland, W. W. & Johnson, M. J., *J. biol. Chem.*, **208** (1954), 679.
11. Damodaran, M., Rangachari, P. N. & Ramakrishnan, K. V., *J. sci. industr. Res.*, **7B** (1948), 29.
12. Vakil, J. R., *Studies on the Metabolism of Aspergillus niger*, Ph.D. Thesis, University of Bombay, 1958.
13. Mokrash, L. C., *J. biol. Chem.*, **208** (1954), 55.
14. Natelson, S., Pincus, J. B. & Lugovoy, J. K., *J. biol. Chem.*, **175** (1948), 745.
15. Bhattacharyya, P. K., *J. sci. industr. Res.*, **17B** (1958), 467.
16. Das Gupta, A. K. & Nair, C. R., *J. sci. industr. Res.*, **15B** (1956), 1.
17. Werkman, C. H. & Wood, H. G., *Advanc. Enzymol.*, **2** (1942), 135.
18. Ochoa, S., *The Enzymes*, Part 2, edited by J. B. Sumner & K. Myrbach (Academic Press, New York), 1951, 929.
19. Ramakrishnan, C. V., *Enzymologia*, **17** (1954), 169.
20. Woronick, C. L. & Johnson, M. J., *J. biol. Chem.*, **235** (1960), 9.
21. Ramakrishnan, C. V., Steel, R. & Lentz, C. P., *Arch. Biochem. Biophys.*, **55** (1955), 270.
22. Ogston, A. G., *Nature, Lond.*, **162** (1948), 963.
23. Schartz, P. & Carter, H. E., *Proc. nat. Acad. Sci., Wash.*, **40** (1954), 499.
24. Hirschmann, H., *Essays in Biochemistry*, edited by S. Graff (John Wiley & Sons Inc., New York), 1955, 156.
25. Fischer, H. F., Frieden, C., McKinley McKee, J. S. & Alberty, R. A., *J. Amer. chem. Soc.*, **77** (1955), 4436.
26. Englard, S. & Colowick, S. P., *Science*, **121** (1955), 866.
27. Massey, V. & Singer, T. P., *J. biol. Chem.*, **228** (1957), 263.
28. Kornberg, H. L. & Krebs, H. A., *Nature, Lond.*, **179** (1957), 988.
29. Marcus, A. & Velasco, J., *J. biol. Chem.*, **235** (1960), 563.
30. Ganguly, N. C. & Chakravarty, K., *J. Amer. chem. Soc.*, **83** (1961), 2581.
31. Seaman, G. R., *J. biol. Chem.*, **228** (1957), 148.
32. Seaman, G. R. & Naschke, M. D., *J. biol. Chem.*, **217** (1955), 1.
33. Wong, D. T. O. & Ajl, S. J., *J. Amer. chem. Soc.*, **78** (1956), 3230; Yukio, Y. & Beevers, H., *Biochem. biophys. Acta*, **48** (1961), 20.
34. Kaziro, Y., Leone, E. & Ochoa, S., *Proc. nat. Acad. Sci., Wash.*, **46** (1960), 1319.
35. Flavin, M. & Ochoa, S., *J. biol. Chem.*, **229** (1957), 965.
36. Ochoa, S. & Kaziro, Y., *Fed. Proc.*, **20** (1961), 982.
37. Wells, P. A., Moyer, A. J. & May, O. E., *J. Amer. chem. Soc.*, **58** (1936), 555.
38. Damodaran, M., Jagannathan, V. & Kartar Singh, *Enzymologia*, **17** (1955), 199.

Role of Carbon Dioxide in Citric Acid Fermentation: Part II—Effect of Carbon Dioxide Tension on the Production of Citric Acid*†

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Withdrawal of carbon dioxide by different methods from the atmosphere above growing *Aspergillus niger* cultures decreases the rate of citrate synthesis and the yield of citrate based on metabolized sugar without materially affecting the growth of the mould and utilization of sugar. Small excesses of atmospheric carbon dioxide, on the other hand, have a remarkable accelerating effect on the growth of the mould and citrate synthesis. This 'carbon dioxide effect' is non-stoichiometric and disappears with increasing concentrations of carbon dioxide. The conclusions have been drawn that (i) carbon dioxide takes part at a stoichiometric level in citrate biogenesis, CO_2 fixation occurring to a significant extent in the process and (ii) traces of carbon dioxide are of fundamental importance in the process of growth of the mould.

IN an earlier communication[1] of this series two major metabolic pathways were outlined to account for incorporation of labelled carbon dioxide into citrate (Charts I and II in ref. 1). In pathway No. 1, the formation of citrate was accounted for by two consecutive processes. Carbon dioxide was fixed to a C_3 fragment, phosphoenolpyruvate or its equivalent, giving rise to a C_4-dicarboxylic acid such as oxalacetate, which was visualized to undergo a subsequent condensation with a C_2 fragment (Chart I in ref. 1).

In pathway No. 2 (Chart II in ref. 1), C_3 fragments were postulated to decarboxylate to C_2 fragments and carbon dioxide. Two of these C_2 fragments were implicated in the formation of a suitable C_4-dicarboxylic acid capable of undergoing a subsequent condensation with another two-carbon fragment to yield citrate.

It was pointed out that labelled carbon dioxide may appear in citrate by (i) a net fixation as in pathway No. 1 and (ii) by an exchange mechanism which could be operative in either of the pathways and as long as there is the possibility of exchange the amount of incorporation can no more be regarded as a criterion on which a choice can be made between the two pathways. The only reliable criterion which could indicate the existence of pathway No. 1 was the molar yield of citric acid, provided it was more than 66·7 per cent[1,2].

It was, therefore, considered desirable to investigate whether any other simple method could be employed to ascertain the major metabolic pathway leading to citrate.

From an examination of both the pathways it appears that if pathway No. 1 were operative to any significant extent, a variation of carbon dioxide tensions above the fermenting mould would be expected to influence the rate of citrate synthesis from the principles of mass action. In pathway No. 2, on the other hand, a variation of the tension of carbon dioxide is not likely to have any appreciable effect on the fermentation.

Preliminary investigations in this laboratory[3] had indicated that removal of carbon dioxide from the atmosphere above the fermenting mould by suspending a test tube containing solid potassium hydroxide resulted in decreased acid production. Erkama *et al.*[4] found that aeration decreased the yield of citric acid in *A. niger*. Some data are also available on the influence of increased carbon dioxide tensions on citrate synthesis. Halliwell[5] reported that passing air containing 2 per cent carbon dioxide over *A. niger* cultures grown on

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†The work constitutes a part of Dr J. R. Vakil's thesis submitted to the University of Bombay, 1957.

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acetate substantially enhanced the yields of citric acid.

Systematic studies on the effect of carbon dioxide tensions on the rate of citrate synthesis were, therefore, justified.

Experimental procedure

Mould, media, cultural conditions and analytical methods were the same as those described earlier[1,3].

Citric acid production under reduced CO_2 tensions— The strain of *A. niger* was grown in 150 ml. of modified Currie's medium[1] (*p*H 1·8) containing 12 per cent glucose in 1-litre Pyrex Erlenmeyer flasks in four series each consisting of three flasks. In the first series beakers containing 50 g. of potassium hydroxide sticks were suspended inside the culture flasks from a stopper on top. The stopper also carried outlet and inlet tubes for air. In the second, the air inside the culture flasks was continuously swept out by a gentle, steady stream of CO_2-free sterile air. An Erlenmeyer flask with standard joints (B40), provided with inlet and outlet tubes for air, and sampling tubes extending down to the bottom of the flasks and with their tops plugged with sterile cotton were used for the aeration studies. Aliquots of the fermenting medium were aseptically withdrawn by suction without disturbing the fermentation.

The following assembly was used for aeration. Sterile air was passed through a sulphuric acid bubbler and a tower of KOH pellets to remove moisture and carbon dioxide, humidified by passage through two bubblers in series containing sterile water and allowed to enter the culture flask dispersed through a perforated bulb 1 cm. above the surface of the medium. The outlet from the culture flask was connected in series to three bubblers, two of which contained 25 ml. of 1*N* CO_2-free sodium hydroxide and the last one a half saturated solution of barium hydroxide (25 ml.). A uniform sweeping rate of 2-3 bubbles per second was maintained throughout the experiment by applying suction at the outlet end of the barium hydroxide bubbler. In the third series, where atmospheric air was used for sweeping, the potassium hydroxide tower and the CO_2-receiving bubblers were omitted. The fourth series served as controls.

Aliquots of the fermented medium were removed from the third day onwards at 24 hr intervals from each flask for the determination of total acidity, citric acid and residual glucose. The CO_2-receiving bubblers were also replaced by a new set every 24 hr and the total respired carbon dioxide was estimated gravimetrically as barium carbonate from the pooled contents of the disconnected bubblers.

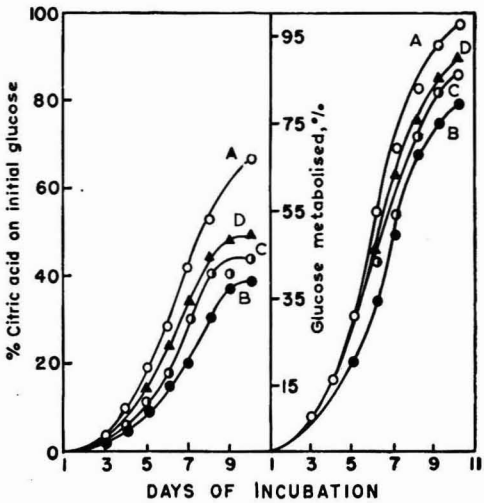

Fig. 1 — Rates of production of citric acid and utilization of glucose under reduced CO_2 tension [(A) Control, (B) alkali beaker, (C) CO_2-free air and (D) atmospheric air]

The rates of production of citric acid on the initial available sugar and glucose utilization are given in Fig. 1.

Removal of carbon dioxide from the culture flasks by any of the methods described above resulted in a decreased rate of citrate formation. The effect was most marked in the first series where alkali beakers were employed to remove carbon dioxide. The maximum yield of citric acid obtained in this case was 40 per cent while a yield of 67 per cent was obtained in the controls. Carbohydrate utilization and growth were not affected to any great extent by removal of carbon dioxide.

It was found, however, that solid potassium hydroxide employed in the first series tended to dehydrate the medium and an almost impervious crust of potassium carbonate appeared at the top of liquefied potassium hydroxide in the beakers with the progress of the fermentation. The growth of the mould was slow, particularly during the first few days of the experiment.

A second experiment was, therefore, designed to remedy these defects. Potassium hydroxide sticks in the beakers were replaced by 20 ml. of 5*N* carbon dioxide-free sodium hydroxide in two series of flasks (three flasks in each series). In the first series alkali was placed in the beakers from the start of the experiment and in the second only after 72 hr when normal mycelial growth had begun. The inlet and outlet tubes of the culture flask were protected from atmospheric carbon dioxide by soda lime tubes. The alkali in the beakers was removed

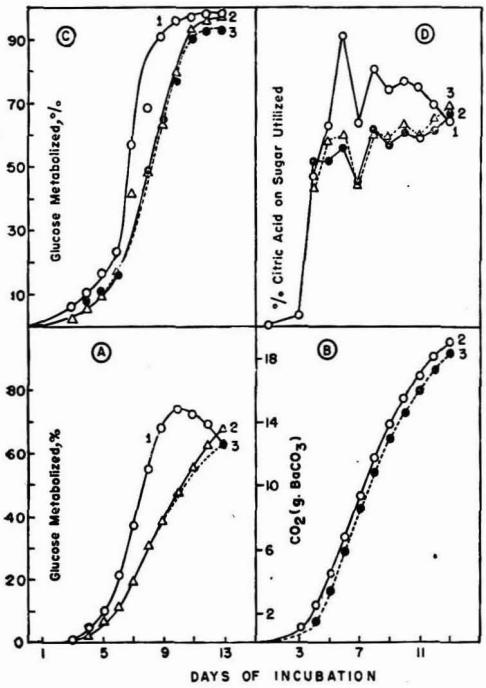

Fig. 2 — Citric acid production under reduced CO_2 tension [(1) Control, (2) NaOH beaker and (3) NaOH beaker kept for 3 days]

daily after the third day onwards for CO_2 determination and replaced by fresh alkali. It was thus possible to get an approximate carbon balance in this experiment.

Citric acid production (on the available sugar), CO_2 evolution, carbohydrate utilization and the yields of citric acid on the metabolized carbohydrate are illustrated in Fig. 2 (A, B, C and D respectively).

The results in the second experiment were not significantly different from those of the first excepting that the ultimate yields of citric acid obtained in the experimental flasks were higher and comparable to those in the controls. From Fig. 2D it is apparent that removal of CO_2 markedly lowered the yields of citric acid on metabolized sugar or in other words there was less utilization of metabolized glucose carbons for citrate synthesis in the absence of CO_2.

Citric acin production under increased CO_2 tension

Since the removal of carbon dioxide was found to retard the formation of citric acid, the effect of increase in the initial carbon dioxide tension in the atmosphere above growing *A. niger* cultures was studied by growing the mould in enclosed systems containing graded amounts of initial CO_2.

Effect of different initial levels of CO_2 on citrate synthesis — Erlenmeyer flasks (Pyrex, 25 ml.) containing 5 ml. of inoculated medium with glucose were placed in 3-litre desiccators (total No. 20). Three flasks were used for each desiccator. The desiccators were arranged in five series of four, designed to contain A, 0·1; B, 0·5; C, 1·0; D, 2·0 per cent CO_2 and E, atmospheric air (0·03 per cent CO_2) respectively. After the inoculated flasks were placed in position, the required carbon dioxide was generated in the enclosed system by tipping in 6 ml. of 18*N* sulphuric acid from a tilted 10 ml.

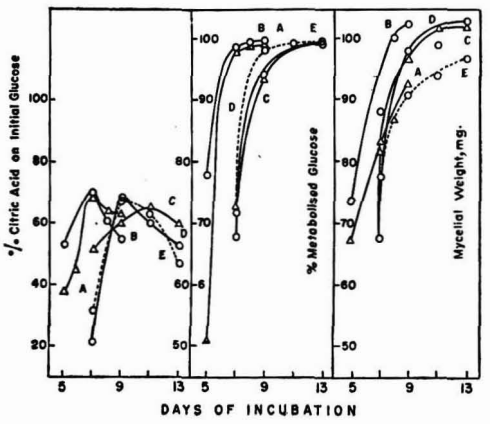

Fig. 3 — Citric acid production under excess CO_2 tension (glucose as C source) [(A) 0·1 per cent CO_2, (B) 0·5 per cent CO_2, (C) 1·0 per cent CO_2, (D) 2·0 per cent CO_2 and (E) control]

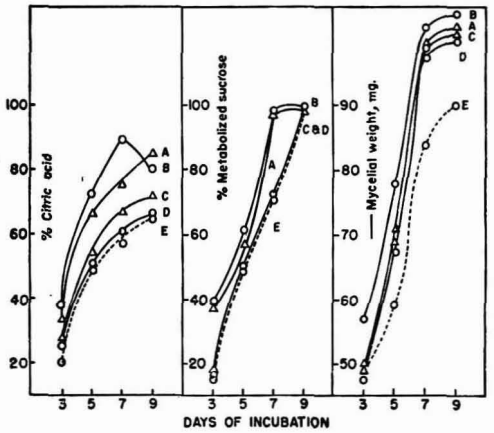

Fig. 4 — Citric acid production under excess CO_2 tension (commercial cane sugar as C source) [(A) 0·1 per cent CO_2, (B) 0·5 per cent CO_2, (C) 1·0 per cent CO_2, (D) 2·0 per cent CO_2 and (E) control]

Erlenmeyer flask into a solution of the required amount of sodium carbonate in 3 ml. of distilled water placed at the bottom of the desiccator. In the controls, CO_2-free distilled water was used in place of aqueous sodium carbonate. The culture flasks were withdrawn at suitable intervals from one of each series of desiccators for analysis.

Fig. 3 represents respectively the progress of acid production, sugar utilization and growth of *A. niger*. It is apparent from the figure that initial carbon dioxide levels from 0·1 to 0·5 per cent accelerated the rate of acid production, sugar utilization and growth. Higher CO_2 levels had a retarding effect, probably due to toxicity. The maximum levels of acidity reached comparable levels for all the concentrations of CO_2 employed.

Similar experiments were conducted with commercial cane sugar as the carbon source in place of glucose. The results (Fig. 4, which give the citrate production, sugar utilization and the mycelial growth) are comparable to those obtained with glucose. However, the maximum acidity reached in this case with all levels of initial CO_2 attained levels significantly higher than that in the controls. The best results were obtained with 0·5 per cent initial CO_2 (89 per cent citric acid; 99 per cent metabolized sugar and 102 mg. dry mycelium on the seventh day of fermentation, the corresponding figures for control being 57 per cent, 70 per cent and 84 mg. respectively).

It should be mentioned here that even within a short incubation period of 40 hr, the thick, rather compact mycelial felt in the flasks incubated under 0·5 per cent initial CO_2 level can be easily distinguished from that in the control flasks (Fig. 5). (The growth in flasks 1 and 2 was obtained with 0·5 per cent initial CO_2 and that in flasks 3 and 4 with atmospheric air after incubation for 40 hr.)

It was obvious that the remarkable stimulatory effect of a small excess of CO_2 initially contained in the atmosphere in a closed system was not due to mass action, since on a molar basis the total amount of excess CO_2 involved (0·13 m. mole) was insignificant when compared to the substrate (10 m. moles). Moreover, in most of the experiments the mould produced appreciable amounts of CO_2 from the substrate carbons and an excess of CO_2 of the order of 0·1 per cent was not likely to make any difference on the total partial pressure of CO_2 attained after one or two days of growth.

It was presumed, therefore, that the beneficial effect of CO_2 was likely to be more pronounced at the very beginning of the experiment. It was thus necessary to study the effect on the mould of exposure to graded levels of CO_2 for different lengths of time.

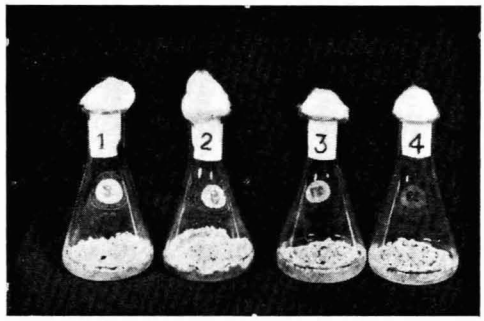

Fig. 5 — Effect of increased CO_2 tension on the growth of mycelium [Flasks 1 and 2 grown under 0·5 per cent CO_2 (40 hr), and flasks 3 and 4 are controls]

The experiment with cane sugar was repeated; this time, however, the lids of the desiccators were removed after 24, 48, 72 and 96 hr, and the gases inside were carefully flushed out with compressed air. The fermentation was then allowed to continue 72 hr with the desiccators which were opened after 24 hr and up to 96 hr in all other cases.

Table 1 summarizes the effect of different period sof exposure to graded levels of CO_2 on citrate synthesis. It is clear from Table 1 that even with a short exposure of 24 hr the stimulatory effect of 0·1 and 0·5 per cent CO_2 was noticeable. The toxic effect of higher levels of CO_2 (1 and 2 per cent) was also apparent as prolonged exposure to these levels led to decreased yields of citric acid.

Discussion

Withdrawal of carbon dioxide results in a substantial reduction in the rate of citrate synthesis. The ultimate yields of citric acid attained on initial available sugar were not affected to any great extent. There is a marked decrease of the yields of citric acid on the basis of utilized sugar. From these data the logical conclusion can be drawn that pathway No. 1 is a significant mechanism of citrate biogenesis under the experimental conditions. It should be pointed out that it is not possible to exclude carbon dioxide completely from the system and both the synthesis of citrate and evolution of carbon dioxide are intracellular processes. Even a complete removal of carbon dioxide from the surrounding atmosphere may not necessarily alter to any great extent the size of the metabolic carbon dioxide pool inside the mycelium. Assuming, therefore, that pathway No. 1 is the only mechanism operating in the mycelium, it is not possible to

TABLE 1 — EFFECT OF EXPOSURE TO DIFFERENT INITIAL LEVELS OF CO_2 ON CITRIC ACID FERMENTATION

(Substrate, commercial cane sugar; fermentation period, 96 hr, unless otherwise specified; temp., 28°±0·5°C.)

Initial level of CO_2	Exposure period in enclosed systems:	Yield of citric acid %				Sugar utilization %				Growth (mycelial wt) mg.			
		24*	48	72	96	24*	48	72	96	24*	48	72	96
A, 0·1%		40·23	59·9	62·0	65·3	56·6	69·3	77·4	79·0	60·0	72·8	79·0	84·0
B, 0·5%		46·80	62·0	64·2	70·2	64·7	77·5	80·6	81·8	65·0	80·3	88·0	94·0
C, 1·0%		33·80	57·0	51·8	47·3	52·6	53·3	51·8	50·5	57·3	66·0	70·3	74·2
D, 2·0%		32·10	56·0	42·5	38·7	45·5	51·5	49·8	49·5	56·7	64·0	68·0	71·0
E, control (atmospheric air; 0·03% CO_2)		28·85	54·4	53·0	54·4	42·6	47·8	50·0	50·2	50·0	56·0	58·7	62·3

*Results after incubation for 72 hr; each reading is an average of triplicates; maximum variation in individual flasks ±4 per cent.

stop citrate synthesis altogether by a mere removal of the atmospheric carbon dioxide.

Unfortunately, these experiments suffer from the same disadvantage as those with $C^{14}O_2$ (ref. 1) in that they cannot prove or disprove the existence of pathway No. 2 in *A. niger*. The data in Fig. 2D are not incompatible with the hypothesis that under decreased carbon dioxide tensions, the C_2-C_2 mechanism[1,6] may play a more prominent role than under normal conditions. The yields of citric acid on utilized sugar under reduced CO_2 tensions do not exceed the limit imposed by pathway No. 2* (ref. 1; 66·7 per cent molar yields).

Since the removal of carbon dioxide from the atmosphere above growing *A. niger* cultures resulted in a reduction in the rate of citrate synthesis, it was expected that increasing the CO_2 tensions above the fermenting culture should favour citrate production. In the experiments conducted with graded levels of excess of carbon dioxide, this speculation was not borne out in the anticipated manner. An unexpected and rather striking stimulation of the metabolism and growth of the mould and an accelerated rate of citrate synthesis were observed with a small excess of carbon dioxide. This is not likely to be a mass action effect since on a molar basis the amount of excess carbon dioxide involved is of the order of 1 per cent of the total carbohydrate metabolized. Further studies from this laboratory[7] as well as by Yanagita[8] have conclusively established that the 'carbon dioxide effect' is more fundamental in nature and is connected with the process of spore germination.

*The mycelial weights were not taken into account in arriving at Fig. 2D. Even if they are corrected for, the basic conclusion remains the same.

The stimulatory effect of carbon dioxide seems to disappear with higher levels of initial carbon dioxide presumably due to the toxicity of the gas. The observed inhibitory effect of carbon dioxide is in conformity with an observation made by Brown four decades ago[9]. He also found that the inhibitory concentrations of carbon dioxide vary to some extent with the fungus and the medium used for growth. The growth of *Botrytis cinera* is inhibited with 20-30 per cent carbon dioxide in water but with 50 per cent in a nutrient medium.

From the isotopic tracer studies reported earlier[1] and the current investigation, it may be concluded that carbon dioxide has two distinct roles in citric acid fermentation: (i) Carbon dioxide participates directly in the synthesis of citrate on a stoichiometric level and that some net fixation of carbon dioxide occurs in citrate; and (ii) traces of carbon dioxide are of fundamental importance in the vital processes of spore germination and growth of the mould.

References

1. Bhattacharyya, P. K., Vakil, J. R., Das Gupta, A. K. & Damodaran, M., *J. sci. industr. Res.*, **21C** (1962), 193.
2. Wells, P. A., Moyer, A. J. & May, O. E., *J. Amer. chem. Soc.*, **58** (1936), 555.
3. Damodaran, M., Rangachari, P. N. & Ramakrishnan, K. V., *J. sci. industr. Res.*, **7B** (1948), 29.
4. Erkama, J., Heikkinen, I., Hagerstand, B. & Junkkonen, S., *Acta chem. Scand.*, **3** (1949), 858.
5. Halliwell, G., *J. exp. Bot.*, **4** (1953), 369.
6. Foster, J. W. & Carson, S. F., *J. Amer. chem. Soc.*, **72** (1950), 1865.
7. Vakil, J. R., Raghavendra Rao, M. & Bhattacharyya, P. K., *Arch. Mikrobiol.*, **39** (1961), 53.
8. Yanagita, T., *Arch. Mikrobiol.*, **26** (1957), 329.
9. Brown, W., *Ann. Bot., Lond.*, **36** (1922), 257.

Nutritive Value of Field Bean (*Dolichos lablab*): Part IV—Effect of Feeding Raw Field Bean on Liver Constituents & Liver Enzymes of Albino Rats

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The livers of rats fed on raw field bean diet show a depletion in glycogen, glutathione, FAD and free riboflavin contents and an increase in fat content. A decrease in the activities of liver enzymes is also observed. Zonal necrosis of liver is observed in rats fed on raw field bean diet but not in those fed autoclaved bean diet. These observations lead to the conclusion that, apart from amino acid deficiency, there is an unknown factor in the field bean which is responsible for liver necrosis and the changes in liver constituents.

IT is well known that liver injury can be initiated by dietary factors alone[1], and changes in the level and quality of dietary protein result in marked changes in the concentrations of liver constituents and the activities of the enzyme systems present in it[2-7]. As raw field bean contains low quality protein and does not support the growth and normal maintenance of rats[8,9], it was considered of interest to study the effect of feeding raw field bean on the various liver constituents and liver enzyme systems of rats.

Experimental procedure

Young male albino rats (35-45 g.) were placed in separate cages with screened bottom. Water was supplied to them *ad libitum*. The rats, 12 in number, were divided into two groups which received (i) casein diet and (ii) raw field bean diet respectively by the pair feeding method. The composition of the diet was: protein, 10; sugar, 31·2; salt mixture[10], 4; groundnut oil, 6 per cent and the total was made to 100 per cent by the addition of corn starch. Half a drop of adexoline, containing 1200 I.U. vitamin A and 200 I.U. vitamin D per rat, was added to the diet. Vitamin mixture[11] (60 mg.) was fed to each rat before feeding the diet. After 13-14 days of experimental period, when the maximum deleterious effects of feeding raw field bean were observed[8], the pairs of rats were sacrificed, the livers dissected out immediately, pressed in a fold of filter paper to remove blood and chilled in an ice tray. Small amounts of the tissue were used for the estimations. Estimations of two constituents of enzyme systems were carried out with one batch of rats. The liver tissue was examined for moisture, protein, non-protein nitrogen, fat, glycogen[12], total SH compound[13], flavine adenine dinucleotide (FAD) and free riboflavin[14], nucleic acids (DNA)[15] and RNA[16], succinic acid dehydrogenase[17], and lactic acid dehydrogenase[17], xanthine oxidase[18] and transaminase[19].

Results and discussion

The results presented in Table 1 indicate that there is no marked difference in moisture, protein, non-protein nitrogen and DNA and RNA contents of the livers of rats fed casein and raw field bean diets. Rats fed raw field bean diet showed accumulation of fat in the liver. Chitre and Vallury[2] have observed that feeding low quality proteins at 10 per cent level in the diet to young rats results in the increased amount of fat in the liver. The accumulation of fat in the liver is also due to the deficiencies of essential amino acids[20-22]. Raw field bean has been found to be deficient in almost all essential amino acids[23]. Nitrogen balance studies have indicated that it contains a protein of low biological value[9] and hence the accumulation of fat in the liver of rats fed raw field bean is observed.

The glycogen content in the livers of rats is depleted when they are fed raw field bean diet. Inamdar and Sohonie[24] have reported similar findings when they fed raw double bean (*Vicia faba* Moench) to rats. Wang *et al.*[25] have reported an increase in the glycogen content of liver of rats fed

TABLE 1 — EFFECT OF FEEDING RAW FIELD BEAN ON CONSTITUENTS AND ACTIVITIES OF ENZYME SYSTEMS IN LIVERS OF RATS

	Casein diet	Raw field bean diet
Moisture, g./100 g. liver	73·00±0·65	74·68±1·06
Protein, g./100 g. dry liver	75·59±0·98	73·82±0·30
Non-protein nitrogen, g./100 g. dry liver	1·81±0·09	1·95±0·08
Fat*, g./100 g. liver	6·35±0·13	10·59±0·26
Glycogen*, g./100 g. dry liver	6·08±0·45	2·08±0·09
Glutathione*, mg./100 g. liver	139·80±2·30	64·35±2·40
FAD*, mg./100 g. dry liver	1·31±0·12	0·57±0·01
Free riboflavin*, mg./100g. dry liver	2·39±0·19	1·40±0·20
DNA, g./100 g. liver	0·27±0·01	0·24±0·06
RNA, g./100 g. liver	1·73±0·09	1·68±0·07
Xanthine oxidase*, µmoles xanthine disappeared/ hr/g. of liver	12·17±0·33	7·68±0·40
Transaminase* QT[10]	286·90±2·70	129·90±3·60
Succinic acid dehydrogenase (time taken to decolourize methylene blue), min.	9	>120
Lactic acid dehydrogenase (time taken to decolourize methylene blue), min.	12	>120

*Denotes the significant difference in two values (casein and raw field bean) when compared statistically at 5 per cent level of significance[38].

on low protein diet. Chitre and Vallury[2] have shown that feeding low quality protein results in an increase in the glycogen content and decrease in the protein content of liver. In the case of rats fed raw field bean diet, the glycogen content of liver is decreased, while the protein content is not affected. As both the diets were fed by pair feeding method, the possibility of the effect of feeding low protein diet does not arise. Raw field bean may contain an unknown factor which affects liver glycogen level in rats.

Glutathione level of the livers of rats fed raw field bean is significantly lower as compared to the livers of casein group rats. Inamdar and Sohonie[24] have observed low glutathione content in the livers of rats fed raw double bean as compared to casein. The level of glutathione in the livers of rats depends entirely upon the sulphur amino acids in the diet[4,26,27]. Methionine is the most limiting amino acid of raw field bean[23]. The deficiency of methionine in the diet may be the cause of appreciable decrease in the glutathione levels in the livers of rats.

The amounts of free riboflavin and FAD are also appreciably low in the livers of rats fed raw field bean. Inamdar and Sohonie[24] have reported similar observations in the case of raw double bean. Seifer *et al.*[28] have reported a decrease in the concentrations of riboflavin in the livers when the rats received protein-free diet. The levels of riboflavin and niacin are directly related to the concentrations of liver nitrogen. Surret and Perlzweig[3] and Riesen *et al.*[29] have also reported similar observations. In the present investigation, as casein and raw field bean diets are fed to rats by pair feeding method, the levels of the liver nitrogen of rats fed on casein and raw field bean diets are comparable, while there is significant difference in their free riboflavin and FAD levels. This effect may be attributed to the low quality of field bean protein or to the presence of an unknown factor in field bean.

The activities of succinic and lactic acid dehydrogenases are low in the livers of rats fed raw field bean diet. Inamdar and Sohonie[24] have also reported similar observations in the case of rats fed raw double bean (*Vicia faba* Moench). The decrease in the activities may be due to the low quality of field bean protein.

Weisterfeld and Richert[30] have observed that the livers with low xanthine oxidase activity are very pale in colour, while very active livers are much darker. In the present investigation, the livers of rats fed raw field bean were pale in colour while those of casein group rats were darker. Litwack *et al.*[31] have reported that low xanthine oxidase activity of livers is partly due to low concentration of FAD in the liver. Williams and Elvehjem[32,33] have shown that deficiency of tryptophan or methionine in the diets markedly reduces xanthine oxidase activity of the livers of rats. Van Pilsum *et al.*[34] have observed that diets deficient in tryptophan, isoleucine or phenylalanine caused a decrease in the xanthine oxidase activity. The loss of FAD in the livers of rats fed raw field bean may be one of the causes of low xanthine oxidase activity. Raw field bean is deficient in methionine, tryptophan, isoleucine, phenylalanine and other essential amino acids. The decrease in the xanthine oxidase activity of livers may be due to the deficiencies of these amino acids in raw field bean diet.

The activity of transaminase in the livers of rats fed raw field bean is decreased as compared to that in livers of casein group rats. The effect may be attributed to low quality protein of field bean or the presence of an unknown factor in raw field bean.

Histological study of liver sections revealed that livers of rats fed raw field bean exhibit zonal necrosis. Such necrosis was not observed in the case of rats fed field bean autoclaved for 5 min. or autoclaved field bean supplemented with essential amino acids. Zonal necrosis was also observed in the case of rats fed raw field bean diet supplemented with essential amino acids.

Schiff[35], Himsworth and Glynn[36] and Daft *et al.*[37] have reported that the deficiencies of sulphur amino acids in the diet cause necrosis of livers. Raw field bean is highly deficient in methionine[23]. However, necrosis was also observed in the livers of rats fed raw field bean diet supplemented with essential amino acids. The livers of rats fed autoclaved field bean, with or without supplementation of essential amino acids, were normal, and their histological pattern was similar to the livers of rats fed casein diet.

All these observations lead to the conclusion that, apart from amino acid deficiency, there is an unknown factor in the field bean, which is responsible for liver necrosis and the changes in liver constituents. This factor appears to be destroyed by autoclaving the beans for 5 min.

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References

1. Weicheselbaum, T. E., *Quart. J. exp. Physiol.*, **25** (1935), 363.
2. Chitre, R. G. & Vallury, S. M., *Indian J. med. Res.*, **44** (1946), 565.
3. Surret, H. P. & Perlzweig, W. A., *J. Nutr.*, **25** (1943), 173.
4. Leef, G. & Neuberger, A., *Biochem. J.*, **41** (1947), 280.
5. Litwack, G., Williams, J. N. (Jr), Chen, L. & Elvehjem, C. A., *J. Nutr.*, **47** (1952), 299.
6. Lightbody, H. D. & Kleinman, A., *J. biol. Chem.*, **129** (1939), 71.
7. Miller, I. L., *J. biol. Chem.*, **172** (1948), 113.
8. Phadke, K. & Sohonie, K., *J. sci. industr. Res.*, **21C** (1962), 155.
9. Phadke, K. & Sohonie, K., *J. sci. industr. Res.*, **21C** (1962), 178.
10. Hegsted, D. M., Milb, R. C., Elvehjem, C. A. & Hart, E. B., *J. biol. Chem.*, **138** (1941), 459.
11. Schultz, M. O., *J. Nutr.*, **41** (1950), 103.
12. Montegomery, R., *Arch. Biochem. Biophys.*, **67** (1957), 378.
13. Grunert, R. R. & Phillips, P. N., *Arch. Biochem. Biophys.*, **30** (1951), 217.
14. Bessay, O. A., Lowry, O. H. & Love, R. H. L., *J. biol. Chem.*, **180** (1949), 755.
15. Dische, Z. & Schwarz, K., *Mikromchim. Acta*, **2** (1933), 13.
16. Brown, A. H., *Arch. Biochem.*, **11** (1946), 269.
17. Thunberg, T., *Scand. Arch. Physiol.*, **40** (1920), 11.
18. Litwack, G., Bothwell, J. W., Williams, J. N. (Jr) & Elvehjem, C. A., *J. biol. Chem.*, **200** (1953), 303.
19. Tonhazy, N. H., White, N. G. & Umbreit, W. W., *Arch. Biochem.*, **28** (1950), 36.
20. Harper, A. E., Monson, W. J., Benton, D. A. & Elvehjem, C. A., *J. Nutr.*, **50** (1953), 383.
21. Benton, D. A., Harper, A. E., Spivey, H. E. & Elvehjem, C. A., *Arch. Biochem. Biophys.*, **60** (1956), 156.
22. Singal, S. A., Hazan, S. J., Sydrenstricker, V. P. & Little John, J. M., *J. biol. Chem.*, **200** (1953), 867, 883.
23. Phadke, K. & Sohonie, K., *J. sci. industr. Res.*, **21C** (1962), 182.
24 Inamdar, A. N. & Sohonie, K., *Ann. Biochem.*, in press.
25. Wang, C. F., Hegsted, D. M., Lapi, L., Zamche, K. N. & Black, M. B., *J. Lab. clin. Med.*, **340** (1949), 953.
26. Beck, L. V. & Bianconi, A. M., *J. Nutr.*, **60** (1956), 197.
27. Jaffe, W. G., *Proc. Soc. exp. Biol., N.Y.*, **97** (1958), 665.
28. Seifer, S., Harkness, D. M., Rubin, R. & Muntwyler, E., *J. biol. Chem.*, **176** (1948), 1371.
29. Riesen, W. H., Schweigert, B. S. & Elvehjem, C. A., *Arch. Biochem. Biophys.*, **10** (1946), 387.
30. Weisterfeld, W. & Richert, D. A., *J. biol. Chem.*, **184** (1950), 163.
31. Litwack, G., Williams, J. N. (Jr), Feigelson, P. & Elvehjem, C. A., *J. biol. Chem.*, **187** (1950), 605.
32. Williams, J. N. (Jr) & Elvehjem, C. A., *J. biol. Chem.*, **183** (1950), 537.
33. Williams, J. N. (Jr) & Elvehjem, C. A., *J. biol. Chem.*, **181** (1949), 559.
34. Pilsum, V., Speyer, J. F. & Samuels, L. T., *Arch. Biochem. Biophys.*, **68** (1957), 42.
35. Schiff, L., *Diseases of Liver* (Pitman Medical Publishing Co. Ltd, London), 1950, 99.
36. Himsworth, H. P. & Glynn, L. E., *Clin. Sci.*, **5** (1944), 93.
37. Daft, F. S., Sebrell, W. H. (Jr) & Lillie, R. D., *Proc. Soc. exp. Biol., N.Y.*, **50** (1942), 1.
38. Snedecor, G. W., *Statistical Methods Applied to Experiments in Agriculture and Biology* (Iowa State College, Iowa), 1940, 179.

Studies on the Metabolic Effects of Aureomycin

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Administration of aureomycin to rabbits diminishes the glucose tolerance in the animals and has a pronounced hypoglycaemic effect. The drug does not alter the glucose tolerance in rats. It decreases serum cholesterol in rats and induces no change in their tissue cholesterol. It diminishes the phospholipid turn over of the tissues.

IT was reported earlier[1,2] that administration of penicillin and dihydrostreptomycin impaired the carbohydrate and lipid metabolism of rabbits and rats. It was also reported[3] that terramycin and achromycin increased cholesterogenesis and phospholipid turn over in the tissues of rats. While the utilization of glucose was diminished after the feeding of terramycin to rabbits and rats, achromycin did not alter the carbohydrate metabolism of these animals[3]. It was observed by Nelson *et al.*[4] that aureomycin augmented hypercholesteraemia of rabbits fed cholesterol. Guggenheim *et al.*[5] reported that aureomycin increased the cholesterol content of liver without any change in serum cholesterol content. The effect of feeding aureomycin, a member of the tetracycline group of antibiotics, on the utilization of sugar and on lipid metabolism was studied and the results are presented in the present communication.

Materials and methods

Healthy male rabbits (1500-2000 g.) were fasted for 16 hr, fed 2 g. glucose per kg. body weight in a 50 per cent solution, blood collected from the marginal ear vein before and at intervals after the administration of glucose and blood sugar determined[6]. The animals were then fed daily 57 mg. aureomycin per kg. body weight for 7 days and on the eighth day glucose tolerance was performed as described above. The drug was withdrawn and after 10 days of withdrawal the glucose tolerance test was repeated. Aureomycin (57 mg./kg.) was fed in a single dose to rabbits fasted for 16 hr. Samples of blood were taken before and at intervals up to 3 hr after the drug was fed, and glucose was estimated in the blood samples. Two groups of adult male rats were fed a basal ration[3]. One of the groups was fed through a stomach tube a daily dose of aureomycin (57 mg./kg.) for 7 days and the other group served as control. Glucose tolerance test was performed in both the groups after the animals were fasted for 16 hr. The animals were sacrificed, tissues were weighed, dried, extracted with petroleum ether in a soxhlet apparatus, and free and ester cholesterol[7], and phospholipid[8] were estimated in the extracts obtained from the different tissues.

Results

Aureomycin in a single dose produced hypoglycaemia in rabbits (Table 1). When administered for 7 days the drug diminished the fasting blood sugar of rabbits but glucose tolerance was diminished at the same time. The sugar tolerance returned to normal level after the withdrawal of the drug for 10 days (Table 2).

Administration of aureomycin in rats neither altered the fasting blood sugar level nor the glucose tolerance. Cholesterol contents of blood serum and of testes diminished after aureomycin administration; the other tissues did not show any alteration in the cholesterol values. There was a significant diminution of the phospholipid content of skin, adrenals, small intestine and kidney of rats administered aureomycin. No significant changes in the phospholipid content of serum, brain, liver and testes were seen in the treated rats. Phospholipid content of the carcass, however, was increased in rats administered aureomycin (Tables 3 and 4).

TABLE 1 — CHANGES IN BLOOD SUGAR LEVEL IN FASTING RABBITS FOLLOWING A SINGLE ORAL DOSE OF AUREOMYCIN (57 MG./KG.)

Rabbit No.	Treatment	Fall in blood glucose from the fasting level (*mg.*/100 *ml.*)			
		45 min.	90 min.	135 min.	180 min.
1	Control (no drug)	7	7	9	13
2	do	1	14	10	10
3	do	7	—	4	5
4	Aureomycin	18	30	44	39
5	do	16	22	33	22
6	do	37	34	—	34

TABLE 2 — GLUCOSE TOLERANCE IN RABBITS BEFORE AND AFTER AUREOMYCIN ADMINISTRATION

(*4 rabbits were used; dose of aureomycin, 57 mg./kg./day for 7 days*)

Condition of animal	Fasting blood glucose *mg./100 ml.*	Rise (+) or fall (−) of blood sugar (*mg./100 ml.*) from the fasting level after the feeding of glucose (*2 g./kg. body wt*)			
		45 min.	90 min.	135 min.	180 min.
Before feeding aureomycin	125±7	+25±3	+40±13	+38±21	−3±11
Seven days after feeding aureomycin	92±7	+68±8	+80±30	+47±22	+16±17
Ten days after withdrawal of aureomycin	120±8	+32±7	+49±28	+22±12	−9±14
	STATISTICAL ANALYSIS (*t* VALUES)				
Between normal and aureomycin treated	3·3*	4·7*	1·2	0·3	0·9
Between normal and after 10 days of withdrawal of aureomycin	0·5	0·8	0·3	0·6	0·9

*These values of *t* only were significant at 5 per cent level.

TABLE 3 — TISSUE CHOLESTEROL OF NORMAL AND AUREOMYCIN-TREATED RATS

[*No. of normal rats, 7; 6 rats were fed aureomycin (57 mg./kg./day, for 7 days); cholesterol values are mean ± standard error expressed as mg./100 g. of fresh tissue (mg. per 100 ml. of serum)*]

Tissue	Normal rats		Aureomycin-fed rats		*t*	
	Total cholesterol	Ester cholesterol	Total cholesterol	Ester cholesterol	Total	Ester
Serum	106±7	55±3	71±3	48±2	4·6*	2·1*
Carcass	217±4	42±5	259±26	59±16	1·5	0·8
Skin	260±20	109±8	286±51	118±24	0·4	0·3
Adrenal	3304±667	2796±649	3645±218	3047±264	0·4	0·3
Small intestine	181±21	71±9	211±27	83±10	0·8	0·8
Brain	2331±163	228±16	2591±146	334±58	1·1	1·7
Liver	320±8	77±15	322±18	109±25	0·5	1·1
Kidney	472±27	110±8	463±34	94±8	0·2	1·4
Testes	288±9	54±5	178±13	19±3	3·0*	3·3*

*These values of *t* only were significant at 5 per cent level.

TABLE 4 — TISSUE PHOSPHOLIPID OF NORMAL AND AUREOMYCIN-FED RATS

(*There were 7 control and 6 treated rats; dose of aureomycin, 57 mg./kg./day for 7 days; phospholipid values expressed in mg. lecithin/100 g. fresh tissue; values are mean ± standard error*)

Tissue	Normal rats	Aureomycin-fed rats	*t*
Serum	139±14	134±13	0·2
Carcass (without skin)	436±23	523±20	2·7*
Skin	415±38	300±38	2·1*
Adrenal	2660±122	1622±191	4·5*
Small intestine	290±13	200±30	2·7*
Brain	3546±177	3721±85	0·9
Liver	1338±65	1172±70	1·7
Kidney	1021±67	717±31	4·1*
Testes	492±33	434±30	1·2

*These values of *t* only were significant at 5 per cent level.

Discussion

Although aureomycin has a hypoglycaemic effect, it diminished the sugar tolerance of rabbits. Sugar tolerance, however, returns to normal after withdrawal of the drug for 10 days. In studies with penicillin[1] it has been observed that the diminished sugar tolerance does not return to normal after withdrawal of the drug for 7 days. In experiments with rats the drug does not show any effect on the utilization of sugar. Of the three tetracyclines, aureomycin and achromycin have the least effect on carbohydrate metabolism of the body. Terramycin markedly disturbs the disposal of blood glucose[3].

Cholesterol content of the body of rats does not increase after aureomycin treatment. There is diminution in the cholesterol of serum of rats treated with the drug. This action is quite in contrast with the effect of other tetracyclines, terramycin and achromycin[3]. No significant change in the liver cholesterol has been observed and a marked diminution of serum cholesterol is observed after the administration of the drug. These observations are contrary to those of Guggenheim *et al.*[5].

Phospholipids content of most of the tissues of rats diminishes after treatment with aureomycin. The administration of terramycin and achromycin, however, increases the tissue phospholipid content and tissue cholesterol contents. It, therefore, seems that of the three tetracyclines studied, aureomycin has no deleterious effect on lipid and carbohydrate metabolism of the body.

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References

1. CHATTERJEE, K. P., SINHA, K. K. & BANERJEE, S., *J. sci. industr. Res.*, **18C** (1959), 110.
2. CHATTERJEE, K. P., SINHA, K. K. & BANERJEE, S., *J. sci. industr. Res.*, **18C** (1959), 126.
3. BANERJEE, S., PRASANNAN, K. G. & GHOSH, P. K., *J. sci. industr. Res.*, **21C** (1962), 13.
4. NELSON, D., IVY, A. C. & ALTSCHUL, R., *Arch. Path.*, **56** (1953), 262.
5. GUGGENHEIM, K., ILAN, J. & PERETZ, E., *J. Nutr.*, **72** (1960), 93.
6. HAGEDORN, H. C. & JENSEN, B. N., *Biochem. Z.*, **137** (1923), 92.
7. SOBEL, A. E. & MAYER, A. M., *J. biol. Chem.*, **157** (1945), 255.
8. YOUNGBURG, G. E. & YOUNGBERG, M. V., *J. Lab. clin. Med.*, **16** (1930), 1958.

Physico-chemical Studies on Indigenous Seed Proteins: Part VII — Electrophoretic Characterization & Amino Acid Composition of Black Gram (*Phaseolus mungo*) Meal Proteins & Its Globulin Fractions

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Optimum electrophoretic separation of black gram meal protein components occurs between *p*H 8·0 and 9·0 and ionic strength 0·1, when four components are obtained. Both ammonium sulphate and combined sodium and ammonium sulphate fractionation procedures give electrophoretically homogeneous globulin fractions. The globulin fraction obtained with the former method has its isoelectric point around *p*H 5·0 and that with the latter procedure around *p*H 5·4. The amino acid analysis of the meal and the fractions revealed the absence of histidine and presence of cystine and methionine in low amounts, while the other essential amino acids are present in substantial amounts.

IN continuation of our studies on the physico-chemical aspects of indigenous seed proteins, the proteins of black gram (*Phaseolus mungo*), one of the popular edible pulses mostly grown in India and South Asian countries, have been analysed.

Seed proteins of a number of species belonging to the genus *Phaseolus* have been investigated by physico-chemical methods. Danielson[1] carried out extensive ultracentrifugal investigations on the proteins of *P. coccineus*, *P. vulgaris* and *P. nanus*. Djang *et al.*[2] carried out detailed solubility studies on *mung* bean (*P. aureus*) meal proteins and also on the electrophoretic characterization of its globulins[3]. In these laboratories, Nath[4] studied the solubility characteristics and electrophoretic properties of green gram (*P. radiatus*) meal proteins and its globulin fractions. However, since no investigations of this kind have been reported on proteins of black gram (*P. mungo*), in the present communication the results on the electrophoretic characterization and amino acid composition of black gram meal proteins and its globulins are presented.

Materials and methods

Black gram meal was prepared by grinding the dehusked seeds and passing through 60 mesh B.S. sieve. The meal was defatted by extensively soxhleting with light petroleum ether.

Standard methods were employed for the determination of total nitrogen, moisture, ash, fat and fibre contents of the meal.

The various fractionation procedures adopted were similar but not always identical with those employed for red gram proteins[5]. Fractionation of the meal proteins was carried out by the following methods.

Fractionation by heat coagulation — Sodium chloride (5 per cent) extract of the meal was heated to different temperatures (60-96°C.) over a water bath. The precipitates formed were removed by centrifugation. One fraction obtained at 68°C. (soluble in buffer, *p*H 8·6) was electrophoretically analysed.

Fractionation by dialysis against different concentrations of sodium chloride — Sodium chloride (1*M*) extract of the meal was dialysed successively against 0·9*M* to 0·01*M* sodium chloride concentrations and the resulting precipitates were analysed electrophoretically.

Ammonium sulphate fractionation — Following the procedure of Jones and Gersdorff[6], sodium chloride (5 per cent) extract of the meal was saturated to various concentrations with ammonium sulphate. All the precipitates obtained were dialysed against distilled water and were then electrophoretically analysed at *p*H 9·0, ionic strength 0·1.

Combined sodium sulphate and ammonium sulphate fractionation — To sodium chloride (5 per cent) extract of the meal an equal volume of 30 per cent sodium sulphate was added at room temperature. The precipitate formed was removed by centrifugation and the supernatant was further fractionated with varying amounts of ammonium sulphate, in the cold. The different fractions obtained were dialysed against distilled water free of salt and then dried in a vacuum desiccator over phosphorus pentoxide, in the cold. All these fractions were analysed electrophoretically. The detailed scheme of this fractionation procedure is outlined in Chart I.

Electrophoretic analyses were carried out by the moving boundary method using the 'Kern' microelectrophoretic apparatus. The composition of buffers used, preparation of extracts and conditions of analysis have been described in earlier studies[7,8].

Amino acid composition of black gram meal and the globulin fractions obtained by combined sodium and ammonium sulphate fractionation procedure was studied by circular paper chromatography. Experimental conditions employed for the preparation of hydrolysates and chromatography were same as described earlier[9,10].

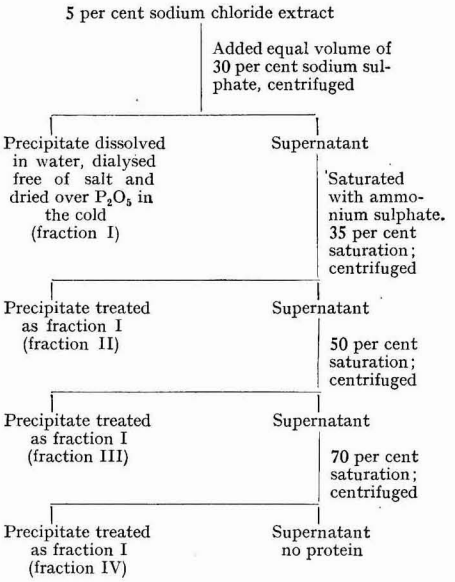


Chart I — Procedure for the fractionation of meal proteins

Results and discussion

The proximate analysis of the meal gave the following percentage values: 3·65 nitrogen, 10·42 moisture, 2·82 ash, 1·84 fibre and 1·64 fat. These values are averages of four independent observations.

The conditions for maximum solubilization of black gram meal were established as follows: (i) 5 per cent sodium chloride, (ii) 60 mesh, petroleum-defatted meal, (iii) ratio of meal to solvent as 1: 10, (iv) mechanical shaking, and (v) 120 min. duration of extraction.

The results of the electrophoretic analyses of meal proteins at various *p*H values are given in Table 1. Optimum separation of the components occurs between *p*H 8·0 and 9·0 and ionic strength 0·1, when four components are obtained. The solubility of proteins below *p*H 5·6 was low and, therefore, analysis could not be carried out very well. At *p*H 4·5 only one diffuse component appeared while at *p*H 5·6, two components appeared in the ascending limb and only one in the descending limb. The separation of protein components improved between *p*H 6·4 and 8·0 when greater number of components were obtained. Complex formation appears to take place among the components beyond *p*H 9·0 also, as evidenced by the decreased number of components. Electrophoretic analysis of the extracts beyond *p*H 10·5 was not possible because of their extreme viscosity. Some of the typical electrophoretic patterns of black gram meal proteins obtained at various *p*H values are shown in Fig. 1.

TABLE 1 — ELECTROPHORETIC ANALYSIS OF BLACK GRAM MEAL PROTEINS AT VARIOUS *p*H VALUES

(*Ionic strength*, 0·1; *temp.*, 25°C.)

*p*H	Mobility $\times 10^5$ cm.^{2} V.$^{-1}$ sec.$^{-1}$							
Components:	I		II		III		IV	
	A	D	A	D	A	D	A	D
4·5	0·47 (100·00)	0·41 (100·00)	—	—	—	—	—	—
5·6	0·52 (80·20)	0·39 (100·00)	—	—	8·02 (19·80)	—	—	—
6·4	0·84 (50·50)	—	5·01 (20·10)	4·48 (70·30)	8·98 (20·90)	7·85 (19·50)	11·47 (8·50)	11·01 (10·20)
7·0	1·32 (45·60)	1·10 (40·80)	5·93 (22·30)	5·19 (30·50)	9·83 (18·10)	9·62 (28·70)	11·68 (14·00)	—
7·4	1·78 (45·00)	2·00 (49·90)	7·58 (30·20)	6·32 (26·70)	12·68 (24·80)	11·69 (23·40)	—	—
8·0	0·83 (34·60)	0·80 (40·50)	6·85 (24·50)	6·21 (20·80)	12·37 (23·70)	11·29 (25·70)	15·75 (17·20)	14·32 (23·00)
8·6	2·20 (30·40)	2·12 (30·80)	7·52 (32·60)	6·83 (40·10)	11·14 (17·20)	10·91 (20·00)	16·19 (19·80)	15·65 (19·10)
9·0	1·34 (37·60)	1·40 (30·60)	6·54 (30·00)	6·61 (28·40	12·08 (20·40)	11·92 (21·00)	16·69 (12·00)	16·34 (20·00)
9·6	2·62 (31·50)	2·43 (68·60)	8·93 (28·20)	9·21 (31·40)	16·95 (25·20)	—	20·31 (15·10)	—
10·5	2·97 (50·80)	3·29 (45·40)	—	—	12·10 (20·90)	11·88 (34·20)	22·28 (28·30)	20·98 (20·40)

Figures in parentheses give percentage distribution of components.
A, ascending limb; D, descending limb.

TABLE 2 — EFFECT OF VARYING IONIC STRENGTH ON THE ELECTROPHORETIC SEPARATION OF BLACK GRAM MEAL PROTEIN COMPONENTS

(*p*H, 8·6; *temp.*, 25°C.)

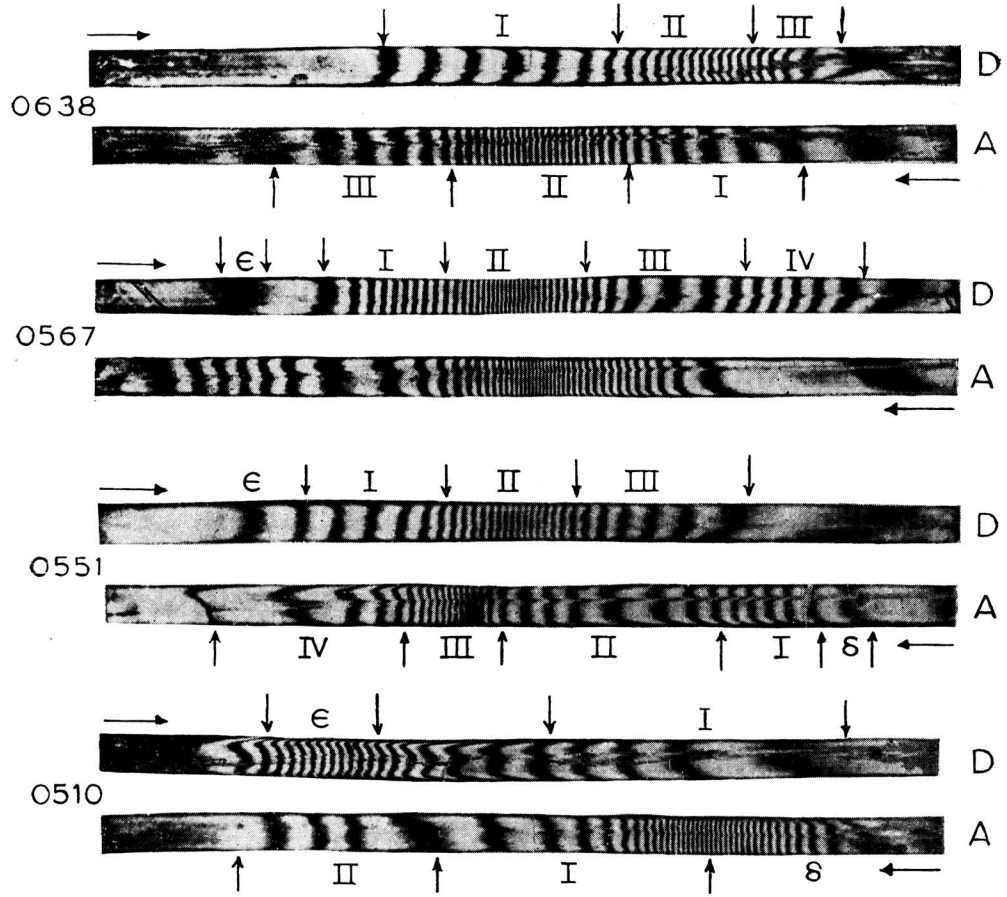
Ionic strength	Mobility $\times 10^5$ cm.^{2} V.$^{-1}$ sec.$^{-1}$							
Components:	I		II		III		IV	
	A	D	A	D	A	D	A	D
0·01	—	—	8·05 (30·60)	7·59 (35·20)	12·53 (69·40)	12·43 (64·80)	—	—
0·05	—	—	8·42 (36·50)	—	11·62 (63·50)	10·56 (75·60)	—	14·82 (24·40)
0·10	2·20 (30·40)	2·12 (30·80)	7·52 (32·60)	6·83 (40·10)	11·14 (17·20)	10·91 (20·00)	16·19 (19·80)	15·65 (19·10)
0·20	3·41 (25·60)	3·02 (20·90)	7·08 (33·30)	6·56 (36·10)	13·42 (41·10)	13·68 (43·00)	—	—
0·50	—	—	5·68 (48·20)	5·02 (55·50)	12·24 (51·80)	11·62 (44·50)	—	—

Figures in parentheses give percentage distribution of components.
A, ascending limb; D, descending limb.

The effect of varying the ionic strength on the electrophoretic separation of black gram meal protein components at *p*H 8·6 is indicated by the results presented in Table 2. As in the case of red gram[8], an ionic strength of 0·1 is most suitable for the optimum separation of protein components. At ionic strengths lower than 0·1 and at 0·5, only two components were observed while at ionic strength 0·2, three components were obtained both in the ascending and the descending limbs.

In coagulation studies, one heavy coagulum was obtained at 68°C. while two minor precipitates were obtained at 84° and 96°C. respectively. The precipitate obtained at 68°C. was soluble in veronal buffer, *p*H 8·6, ionic strength 0·1 and when electrophoretically analysed showed two components (Fig. 2). Other components were insoluble in the buffer, showing denaturation.

The results of the electrophoretic analyses of the fractions obtained by dialysis of 1*M* sodium chloride extract against various concentrations of sodium chloride are given in Table 3. The method of dialysis against different concentrations of sodium chloride has been successfully employed by

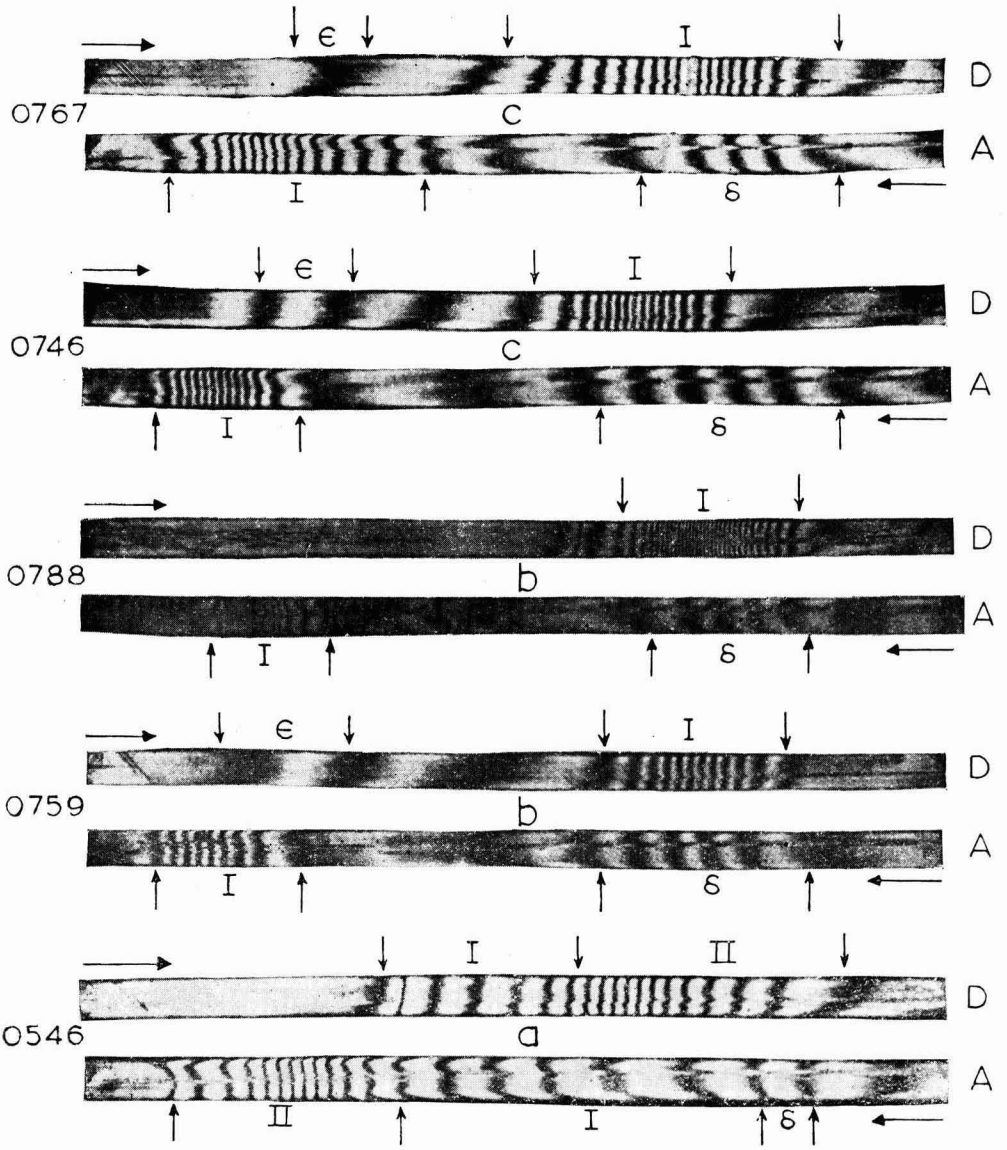


Pattern No.	*p*H	Voltage gradient *V./cm.*	Duration *sec.*	Temp. °C.
0510	5·6	4·5	3900	27·2
0551	7·0	4·1	3300	25·5
0567	8·6	3·6	3000	25·3
0683	10·5	3·9	2700	26·1

Fig. 1 — Typical electrophoretic patterns of black gram meal proteins at various *p*H values and ionic strength 0·1 [A, ascending pattern; D, descending pattern; I, II, III and IV are components]

Djang *et al.*[3] who isolated four homogeneous globulins from *mung* bean. Following the same procedure, Karon *et al.*[11] have obtained an electrophoretically pure fraction from cottonseed proteins. By the application of this procedure in the present study, four precipitates were obtained and the fraction obtained at 0·05-0·01*M* concentration of sodium chloride was electrophoretically homogeneous at *p*H 8·6. However, since this method involved considerable loss of proteins, it was not used for large-scale fractionation.

In Table 4 are given the results of electrophoretic analysis of the four protein fractions obtained by ammonium sulphate fractionation at *p*H 8·6 and ionic strength 0·1. Unlike in the case of red gram proteins[9], an electrophoretically homogeneous fraction could be obtained from black gram proteins by this method. The fraction obtained at 70 per cent ammonium sulphate saturation was found to be homogeneous at *p*H 8·6 and hence was analysed at various *p*H values ranging from 3·0 to 10·0. These results are presented in Table 5



Pattern No.	Sample	*p*H	Voltage gradient *V./cm.*	Duration *sec.*	Temp. °C.
0546	a	8·6	4·2	2700	29·5
0759	b	5·6	4·1	2520	28·2
0788	b	9·0	3·2	2100	26·5
0746	c	5·6	3·8	3000	26·2
0767	c	9·0	3·5	2100	26·0

Fig. 2 — Typical electrophoretic patterns of: (a) coagulum obtained at 68°C.; (b) homogeneous fraction obtained by 70 per cent ammonium sulphate saturation and (c) homogeneous fraction obtained by combined sodium and ammonium sulphate fractionation (fraction III) [A, ascending pattern; D, descending pattern; I and II are components]

TABLE 3 — ELECTROPHORETIC ANALYSIS OF THE FRACTIONS OBTAINED BY DIALYSIS OF 1*M* EXTRACT AGAINST VARIOUS CONC. OF SODIUM CHLORIDE

(*p*H, 8·6; *ionic strength*, 0·1; *temp.*, 25°C.)

Sodium chloride conc. *M*	Components:	Mobility × 10^5 cm.² V.$^{-1}$ sec.$^{-1}$							
		I		II		III		IV	
		A	D	A	D	A	D	A	D
0·91-0·5		—	—	—	—	11·52 (30·20)	11·32 (20·50)	16·46 (69·80)	16·00 (79·50)
0·5-0·1		—	—	7·34 (30·10)	7·42 (25·50)	10·38 (59·40)	11·21 (60·50)	15·48 (10·50)	15·23 (14·00)
0·1-0·05		2·42 (30·40)	2·02 (25·60)	7·63 (69·60)	8·02 (74·40)	—	—	—	—
0·05-0·01		—	—	8·24 (100·00)	7·92 (100·00)	—	—	—	—

Figures in parentheses give percentage distribution of the components.
A, ascending limb; D, descending limb.

TABLE 4 — ELECTROPHORETIC ANALYSIS OF BLACK GRAM PROTEIN FRACTIONS OBTAINED BY AMMONIUM SULPHATE FRACTIONATION

(*p*H, 8·6; *ionic strength*, 0·1; *temp.*, 25°C.)

Ammonium sulphate saturation at which different fractions are obtained %	Components:	Mobility × 10^5 cm.² V.$^{-1}$ sec.$^{-1}$							
		I		II		III		IV	
		A	D	A	D	A	D	A	D
35		—	—	8·42 (20·40)	7·56 (31·60)	11·52 (59·60)	11·05 (68·40)	17·42 (20·00)	—
50		—	—	6·42 (16·50)	6·35 (24·40)	10·42 (83·50)	10·22 (75·60)	—	—
70		—	—	5·72 (100·00)	5·55 (100·00)	—	—	—	—
90		2·92 (34·60)	3·09 (42·30)	7·62 (65·40)	8·02 (57·70)	—	—	—	—

Figures in parentheses give percentage distribution of the components.
A, ascending limb; D, descending limb.

TABLE 5 — ELECTROPHORETIC MOBILITIES OF HOMOGENEOUS FRACTIONS AT DIFFERENT *p*H VALUES

(*Ionic strength*, 0·1; *temp.*, 25°C.)

*p*H	Components:	Mobility × 10^5 cm.² V.$^{-1}$ sec.$^{-1}$			
		Fraction A		Fraction B	
		A	D	A	D
3·0		+5·90	+5·82	+6·23	+6·10
4·0		+3·32	+2·92	+3·72	+3·51
4·5		+1·26	+1·20	+2·40	+2·15
5·0		0·00	0·00	+1·05	+0·95
5·6		−1·92	−1·69	+0·42	+0·40
6·4		−3·85	−3·80	−2·76	−2·41
7·4		−5·22	−5·31	−4·92	−4·45
8·0		−5·75	−5·77	−5·50	−5·40
9·6		−6·56	−6·44	−7·34	−6·85
10·1		−6·79	−6·77	−7·92	−7·29

Fraction A: Fraction obtained by 70 per cent ammonium sulphate saturation of 5 per cent sodium chloride extract of black gram meal.
Fraction B: Fraction III obtained by combined sodium sulphate and ammonium sulphate fractionation.
A, ascending limb; D, descending limb.

and the *p*H-mobility curve is shown in Fig. 3. This fraction moved as a single protein boundary in this *p*H range and from the *p*H-mobility curve, its isoelectric point could be assigned around *p*H 5·0. A few of the typical electrophoretic patterns of this fraction are shown in Fig. 2.

The results of electrophoretic analysis of the various protein fractions obtained by combined sodium and ammonium sulphate fractionation procedure are given in Table 6. Only fraction III was electrophoretically homogeneous at *p*H 8·6, while the other fractions were heterogeneous. Fraction III was analysed at various *p*H values ranging from 3·0 to 10·0 and the results are presented in Table 5 along with the mobility data of the homogeneous fraction obtained by ammonium sulphate fractionation. The *p*H-mobility curve of this fraction is shown in Fig. 3 which indicates its isoelectric point to be around 5·4. It may be recalled that Djang *et al.*[3] have reported the isoelectric points of the four homogeneous fractions they obtained from *mung*

TABLE 6 — ELECTROPHORETIC ANALYSIS OF BLACK GRAM PROTEIN FRACTIONS OBTAINED BY COMBINED SODIUM SULPHATE AND AMMONIUM SULPHATE FRACTIONATION

(*p*H, 8·6; *ionic strength*, 0·1; *temp.*, 25°C.)

Fraction	Mobility $\times 10^5$ cm.^{2} V.$^{-1}$ sec.$^{-1}$							
Components:	I		II		III		IV	
	A	D	A	D	A	D	A	D
I	1·42 (80·30)	1·09 (73·70)	6·42 (19·70)	6·05 (26·30)	—	—	—	—
II	—	—	6·82 (14·10)	7·12 (20·80)	11·46 (50·90)	11·54 (79·20)	17·21 (35·00)	—
III	—	—	7·31 (100·00)	7·22 (100·00)	—	—	—	—
IV	—	—	—	—	12·31 (15·40)	12·03 (20·60)	10·42 (84·60)	18·44 (79·40)

Figures in parentheses give percentage distribution of the components.
A, ascending limb; D, descending limb.

TABLE 7 — AMINO ACID COMPOSITION OF BLACK GRAM MEAL AND THE FRACTIONS OBTAINED BY COMBINED SODIUM AND AMMONIUM SULPHATE FRACTIONATION PROCEDURE

(*Values are expressed in g./100 g. substance and are averages of* 10 *determinations*)

Amino acid	Fraction I	Fraction II	Fraction III	Fraction IV	Meal	
					Exp. values	Lit.[12] values
Alanine	2·74	4·89	3·28	3·61	0·97	0·91
Arginine	2·74	2·80	2·22	2·45	1·27	—
Aspartic acid	8·93	12·22	5·21	4·91	4·02	2·24
Cystine	1·08	2·06	0·64	0·91	0·61	—
Glutamic acid	9·52	8·70	5·55	5·43	3·12	3·37
Glycine	2·86	2·31	1·58	2·56	0·97	1·00
Histidine	—	—	—	—	—	3·07
Isoleucine	4·52	5·72	2·92	2·17	1·14	3·53
Leucine	8·39	8·86	5·28	3·43	1·35	—
Lysine	7·69	8·92	2·34	3·44	2·96	2·90
Methionine	0·44	0·43	0·42	0·41	0·30	—
Phenylalanine	6·30	5·43	2·36	1·44	1·47	1·38
Proline	1·53	3·89	1·59	1·08	0·82	0·97
Serine	3·58	5·76	3·28	2·15	1·33	0·97
Threonine	3·53	3·76	1·46	2·83	0·96	1·00
Tryptophan	0·64	0·72	0·23	0·71	0·31	0·50
Tyrosine	0·42	—	—	1·43	0·70	0·56
Valine	4·56	5·05	2·36	1·76	1·41	1·26

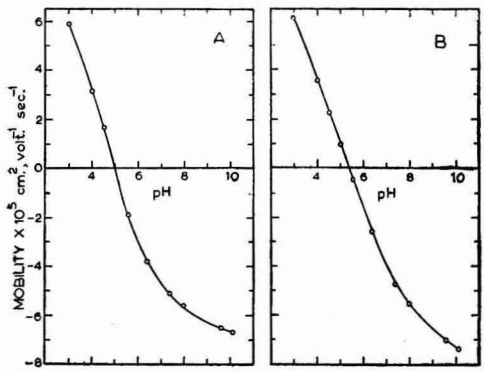


Fig. 3 — *p*H-mobility curve of (A) fraction obtained by 70 per cent ammonium sulphate saturation at ionic strength 0·1 and (B) fraction III obtained by combined sodium and ammonium sulphate fractionation at ionic strength 0·1

bean to be 5·0, 5·2, 5·4 and 5·7. A few of the typical electrophoretic patterns of fraction III are given in Fig. 2. These two fractions remained homogeneous when analysed at different ionic strengths at *p*H 7·0 (phosphate buffer).

In Table 7 are given the amino acid compositions of black gram meal and the four protein fractions obtained by combined sodium and ammonium sulphate fractionation procedure. The amino acid composition reported by Bagchi *et al.*[12] for this meal has been given for comparison since it has been obtained by the method of paper chromatography. The amino acid values obtained in the present study agree fairly well with those reported except for aspartic acid. As is generally observed in leguminous seeds, black gram meal as well as its fractions contain low amounts of cystine and

methionine. Among basic amino acids, arginine is present almost to the same extent in the different fractions and to a lesser extent in the meal. While lysine is present in good amounts in fractions I and II, its content in the other fractions and the meal is more or less similar. Aspartic and glutamic acids are present to a greater extent in fractions I and II.

Among other essential amino acids, as in the case of red gram[10], histidine is absent in the meal and its fractions. Tryptophan is equally distributed in all the fractions except fraction III in which it is present in a lower amount. Leucines are present in good amounts in all the fractions but to a greater extent in fractions I and II. Tyrosine content of black gram meal is low and it is distributed mainly in fractions I and IV, largely in the latter.

There are significant differences in the amino acid composition of individual fractions, but the essential amino acids appear to be present to a greater extent in fractions I and II. A comparison of the amino acid composition of red gram and black gram meals and their fractions shows that the latter is richer in essential amino acids and, therefore, may well be nutritionally superior. In addition, this observation is in accordance with nutritive value data[13].

Acknowledgement

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References

1. Danielson, C. E., *Biochem. J.*, **44** (1949), 387.
2. Djang, S. S. T., Ball, C. D. & Lillevik, H. A., *Cereal Chem.*, **30** (1953), 230.
3. Djang, S. S. T., Ball, C. D. & Lillevik, H. A., *Arch. Biochem. Biophys.*, **40** (1952), 165.
4. Nath, R., *Physico-chemical Studies on the Interactions of Proteins with Large and Small Organic Molecules*, Doctoral Thesis, Agra Univ., 1957.
5. Tawde, S. & Cama, H. R., *Proceedings of the Symposium on Proteins* (Central Food Technological Research Institute, Mysore), 1960, 8.
6. Jones, D. B. & Gersdorff, C. E. F., *J. biol. Chem.*, **75** (1927), 213.
7. Nath, R. & Giri, K. V., *J. sci. industr. Res.*, **16C** (1957), 5.
8. Tawde, S. & Giri, K. V., *J. sci. industr. Res.*, **19C** (1960), 190.
9. Nath, R., Hanumantha Rao, K. & Giri, K. V., *J. sci. industr. Res.*, **16C** (1957), 228.
10. Tawde, S. & Cama, H. R., *J. sci. industr. Res.*, **21C** (1962), 162.
11. Karon, M. L., Adams, M. E. & Altachul, A. M., *J. phys. Chem.*, **54** (1950), 56.
12. Bagchi, S. P., Ganguli, N. C. & Roy, S. C., *Ann. Biochem.*, **15** (1955), 49.
13. Kuppuswamy, S., Srinivasan, M. & Subrahmanyan, V., *Proteins in Foods* (Indian Council of Medical Research, New Delhi), 1958.

Short Communications

A Novel Type of Enzyme Inhibition

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It is concluded that three factors are involved in the inactivation of oxalic acid oxidase of *Bougainvillea spectabilis* occurring during acetone treatment of the whole leaves: the solvent, the debris fraction derived from the leaves and the plastid-bound enzyme.

THE occurrence of a plastid-associated oxalic acid oxidase in the leaves of *Bougainvillea spectabilis* was reported from this laboratory[1]. It was also stated that acetone drying of whole leaves resulted in inactivation of the enzyme, whereas similar treatment of the separated plastid fraction gave an active preparation. We have now found that cell debris is required as the source of inhibitor, but that actual inhibition results only when the debris and the plastids are brought together in contact with acetone.

The preparation of homogenates and isolation of the particulate fraction have already been described[1]. The cell debris resulting on homogenization of leaves in water was collected by filtering through two layers of muslin. The material subjected to acetone treatment, in the form either of a solid material or an aqueous suspension, was taken in the chilled glass bowl of a Waring blender and ground for 2 min. with about 10 parts of acetone chilled to −18°C. The suspension was filtered under suction on a Buchner funnel and the residue treated again with acetone, the process being

TABLE 1 — ACTIVITY OF DIFFERENT PREPARATIONS

Treatment	Result (in terms of enzyme activity)
1. Acetone powder of whole leaves	Inactive
2. Vacuum dried powder of whole leaves	Activity of fresh leaves fully retained
3. Acetone powder of vacuum dehydrated whole leaves	Inactive
4. Acetone powder of plastid fraction from water homogenate	Active (60-70% of the activity of fresh particles)
5. Acetone powder of plastid fraction + acetone powder of whole leaves	Activity of the former fully recovered
6. Acetone powder of aqueous homogenate after discarding coarse debris	Activity fully retained
7. Acetone powder of aqueous homogenate without removal of coarse debris	Inactive
8. Acetone powder of cellular debris	do
9. Acetone powder of debris-freed homogenate + acetone powder of the debris	Activity of the former fully retained
10. Acetone powder of a mixture of fresh plastid and debris fractions	Inactive
11. Acetone powder of a mixture of fresh plastid fraction and acetone dehydrated debris	Activity of the former fully recovered
12. Acetone powder of plastid fraction + aqueous residue from acetone extract of debris	do

repeated until a preparation practically free from chlorophyll was obtained. The powder was spread in thin layer, allowed to dry at room temperature for an hour and finally *in vacuo* over fused calcium chloride in the cold and stored in a deep-freeze. In other experiments whole leaves were dried *in vacuo* over calcium chloride at 0°C. and powdered. Acetone extracts were distilled under reduced pressure and the resulting aqueous suspension tested for inhibitory activity. The enzyme activity was determined by the manometric technique[1]. The observations are recorded in Table 1.

It is clear from the results given in Table 1 that the inactivation of whole leaves on acetone treatment is not due to dehydration as such, but due to secondary changes induced by the solvent. In order that this inactivation or inhibition may occur, the presence of cellular debris at the time of acetone treatment is essential.

One of us (S.K.S.) is grateful to the Council of Scientific & Industrial Research, New Delhi, for financial assistance.

Reference

1. SRIVASTAVA, S. K. & KRISHNAN, P. S., *Biochem. J.*, **84** (1962), in press.

Removal of Phosphorus from Sewage by Sand Filters

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Most of the phosphorus from sewage during filtration through the soil and sand is found to be quickly removed in the initial stages. This removal was apparently due to the chemical combination of the phosphorus with the reactive iron and aluminium in the filter. In the later stages of filtration, the effect of other changes, presumably due to microbial activity, was more evident in the removal of the phosphorus and this effect was seen to a greater extent in the filter which showed comparatively more organisms such as Vorticella sp.

PHOSPHATES in sewage effluents have, in recent years, been a subject of close study because of their influence on algal blooms in the receiving waters[1-3]. Among the harmful effects of blooms according to Lackey[4] are: (i) they sometimes kill fish in inland waters, apparently through anoxia; (ii) they secondarily kill people along the California coast by producing a poison which accumulates in edible mussels in toxic amounts; (iii) they kill, by some not understood mechanism, cattle which drink from inland ponds; and (iv) they clog filters in water plants.

The recent observation on the rapid removal of most of the phosphorus in sewage by activated sludge[5] and its possible bearing on the auto-inactivation of enteroviruses as a result of depletion of phosphorus, a factor in virus survival in water[6], suggested an examination of the nature and extent of removal of phosphorus from sewage during filtration through materials such as sand and granite chips, about which the available evidence is inadequate[7-12]. As filters are widely used for the treatment of sewage, information on how much of the sewage phosphorus is 'fixed' or retained in such filters, how much of it, including the water-soluble phosphorus, comes out in course of time and the factors influencing these changes would be of interest. On these and related aspects some experimental work has been carried out and the results of this work are presented in this communication.

Samples (250 ml.) of detritus-free domestic sewage were allowed to percolate through one-foot columns of granite chips of ½ in. size, Bangalore red soil, clay soil from a rice field, sand from a river bed and sand fractions which passed through and retained on mesh-sieves of 10-20, 20-40, 40-60, 60-80, 80-100 and 100-150. These materials were

taken in glass tubes of 1·75 in. diameter. The bottom portions of these glass tubes were drawn out to form narrow tubes of about 0·3 in. diameter, to which rubber tubings with pinch cocks were attached for regulating the filtration of the liquids at a uniform rate of 20 ml./min. At the point of constriction in each tube a layer of glass wool was placed to hold the filter material. In the first three weeks the sewage was recirculated seven times every day to facilitate the growth of the oxidizing organisms in the filters. After this period of 'ripening' the filtration was carried out once a day for a fortnight. When the filters were rested during the night-time or on holidays, the media were kept moist by keeping a thin layer of water on them.

The effluents were daily analysed for their total and water-soluble phosphorus contents by the method described by Fiske and Subbarow[13] and modified by King[14]. The turbidity of the effluents and the oxygen absorbed from acidified potassium permanganate solution in 3 min. and 4 hr were determined[5], and iron and aluminium oxides in the soils and sewage were determined by the methods described by Wright[15] and Theroux *et al.*[16]. Microscopic examination of the dominant forms of non-bacterial life in the filters was also carried out. The experiment was repeated three times. The results relating to the removal of phosphorus from the sewage by different filters on the 1st, 9th, 20th, 22nd and 34th day are given in Table 1.

The results given in Table 1 indicate: (i) that in the initial stages the granite chips did not remove any phosphorus; (ii) that the quick removal of most of the phosphorus on the 1st day by the soils, sand and sand fractions was due to a chemical reaction, apparently by the combination of the phosphorus with the reactive iron and aluminium in these filter materials; the amounts of iron and aluminium (Fe_2O_3 and Al_2O_3) in the Bangalore red soil, sand and in the sewage were 16·5 per cent, 6·6 per cent and 40·3 p.p.m. respectively (the figure for sewage being the average for the samples used); and (iii) that in course of time the chemical reaction decreased and the effect of other changes presumably due to microbial activity was more evident in the removal of phosphorus, the effect being comparatively more in the '40-60' mesh filter on the 34th day. This filter then contained more Vorticella sp. than the other filters. Also, the effluent from this filter was clearer and more oxidized than the effluents from the other filters, as indicated by the values for turbidity and 3 min. permanganate values.

The clear effluents from the treatment of the sewage with chemical coagulants, such as lime, alum and ferric chloride, did not show any phosphorus. But the clear filtrates obtained by forcing the sewage through an unglazed porcelain tube of $\frac{1}{8}$ in. thickness and $\frac{7}{8}$ in. diameter contained all the water-soluble phosphorus and about 15 per cent of the organic phosphorus of the sewage. When a 6 in. column of the red soil in a glass tube of 1 in. diameter was treated with a 2·5 per cent solution of 8-hydroxyquinoline in acetic acid and thus blocking the iron and aluminium in the soil[17] (the filter was washed free of the acid before the addition of sewage), practically all the phosphorus in the added sewage came out in the effluent; the control column of the soil, which was not treated with 8-hydroxyquinoline, retained all the phosphorus and the effluent did not show any of it. Plot experiments and pot culture studies have indicated that application of superphosphate or potassium phosphate to the red soil under sewage irrigation evokes better response of crops[18].

TABLE 1 — PERCENTAGE REDUCTION IN PHOSPHORUS IN THE SEWAGE PERCOLATED THROUGH VARIOUS FILTERS*

Effluent analysed on — Filter:	Granite chips		Sand		Sand fraction, *mesh size*									
	WSP	TP	WSP	TP	10-20		20-40		40-60		60-80		80-100	
					WSP	TP	WSP	TP	WSP	TP	WSP	TP	WSP	TP
1st day	0·0	0·0	89·0	81·7	77·0	80·0	84·0	84·1	88·0	87·3	95·0	93·6	100·0	96·2
9th day	0·0	0·9	48·2	52·4	41·8	50·8	48·2	52·4	44·7	47·6	41·8	50·0	45·6	47·6
20th day	0·0	13·0	34·0	43·1	38·0	45·5	30·0	40·6	37·0	46·3	47·0	51·2	—	—
22nd day	3·3	10·0	55·6	28·3	46·7	30·8	46·7	33·3	58·9	45·0	46·7	44·1	—	—
34th day	7·5	10·0	29·0	39·1	43·0	50·0	39·8	50·0	61·3	61·7	50·6	61·7	—	—

*The filters containing clay and Bangalore red soil clogged on the 1st day and the filter containing sand fraction of 100-150 mesh size clogged on the 6th day of the experiment. The figures for the percentage reduction in water-soluble and total phosphorus for these filters on the 1st day are 100 and 100, 100 and 100, and 100 and 96·2 respectively. The filter containing sand fraction of 80-100 mesh size clogged on the 19th day of the experiment.

WSP, water-soluble phosphorus; and TP, total phosphorus.

The above observations seem to be of considerable interest and value in controlling the phosphorus contents of sewage effluents to be discharged into water courses and in the study of the processes influencing the changes of phosphorus in the soil.

References

1. SAWYER, C. N., *J. New Engl. Wat. Wks Ass.*, **61** (1947), 109.
2. SMITH, F. G. W., WILLIAMS, R. H. & DAVIS, C. C., *Ecology*, **31** (1950), 119.
3. OHLE, W., *Naturwissenschaften*, **40** (1953), 153.
4. LACKEY, J. B., *Sewage industr. Wastes*, **26** (1954), 326.
5. SRINATH, E. G., SASTRY, C. A. & PILLAI, S. C., *Experientia*, **15** (1959), 339.
6. KELLY, S., SANDERSON, W. S. & NEIDL, C., *J. Water Pollution Cont. Fed.*, **33** (1961), 1056.
7. FRICKE, E., HASELHOFF, E. & KONIG, J., *Landw. Jahrb.*, **22**, 801; *J. chem. Soc. Abstr.*, **66** (1894), 212.
8. MUNTZ, A. & LAINE, E., *C.R. Acad. Sci., Paris*, **152** (1911), 1814.
9. RUDOLFS, W., *Sewage Wks J.*, **19** (1947), 43.
10. BELCHER, E. H., *J. Inst. Sew. Purif.*, Part 3 (1951), 348.
11. HARKNESS, N. & JENKINS, S. H., *J. Inst. Sew. Purif.*, Part 1 (1958), 85.
12. STONES, T., *Sewage industr. Wastes*, **31** (1959), 981.
13. FISKE, C. H. & SUBBAROW, Y., *J. biol. Chem.*, **66** (1925), 375.
14. KING, E. J., *Biochem. J.*, **26** (1932), 292.
15. WRIGHT, C. H., *Soil Analysis: A Handbook of Physical and Chemical Methods* (Thomas Murby & Co., London), 1939, 180.
16. THEROUX, F. R., ELDRIDGE, E. F. & MALLMANN, W. L., *Laboratory Manual for Chemical and Bacterial Analysis of Water and Sewage* (McGraw-Hill Book Co. Inc., New York), 1943, 25.
17. GHANI, M. O., *Indian J. agric. Sci.*, **13** (1943), 562.
18. PILLAI, S. C., RAJAGOPALAN, R. & SUBRAHMANYAN, V., *Investigations on Sewage Farming* (Indian Council of Agricultural Research), 1945, 47; 1946, 36.

A Possible Direct Effect of Thiourea on the Rat Testis

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Direct injection of thiourea evokes certain changes in the rat testis which cannot be imputed to its stereotyped antithyroid activity; altered osmomolarity in the organ due to this mode of administration cannot be held solely responsible, either. These changes are of a degenerative nature affecting mostly the seminiferous tubules. It is possible that thiourea has some direct effect on the testis.

IT has been held from time to time that all of the pharmacologic effects of thiourea are not just secondary manifestations of its basic antithyroid activity. Thus, the nature of adrenocortical changes is not quite the same in hypothyroidism induced either by thiourea or by surgical thyroidectomy[1-4]. On similar grounds, an increase in weight, together with the alterations of succinoxidase and catalase activities in the liver, has been imputed to a direct effect of the compound[5-8]. In view of these facts, it seemed worth while to examine whether the reported effect of thiourea on the rat testis is direct or mediated solely through the thyroid[9,10].

Colony-bred albino rats of the Institute, weighing 140-150 g., were used in the investigation. In the thyroidectomized animals thiourea (or saline) treatment commenced 15 days after the operation. All of the animals were maintained under uniform laboratory conditions throughout the experimental period.

Thiourea was administered intratesticularly under asepsis in a dose of 10 mg./testis (in 0·2 ml. of sterile distilled water) on alternate days for 14 days. In order to eliminate any non-specific effects of altered osmomolarity in the testis, groups of normal and thyroidectomized rats were injected with equimolar amount of sodium chloride (3·56 mg./testis in 0·2 ml. of distilled water) in a similar manner. Thyroxine was administered by the subcutaneous route at the rate of 6 μg./rat on alternate days for 14 days.

The animals were sacrificed on the day following the final injections. The testes were dissected out and weighed to the nearest mg. in a torsion balance. For histological studies the organ was fixed in Bouins fluid and serial paraffin sections were stained with Ehrlich's haematoxylin and eosinol. Total cholesterol concentration of the testis was estimated by the modified method of Zlatkis *et al.*[11,12].

It will be evident from the results presented in Table 1 that the testis weight did not register any significant change between the different experimental groups when subjected to analysis of variance test. However, this was not the case with testicular cholesterol concentration. All of the thyroidectomized groups had significantly lower cholesterol concentration in comparison to that of the normal animals injected with thiourea or

TABLE 1 — EFFECT OF THIOUREA OR SODIUM CHLORIDE ON WEIGHT AND CHOLESTEROL CONCENTRATION OF THE TESTIS

Treatment	Testis weight (*g.*) with S.E.	Testis cholesterol (*mg./g.*) with S.E.
Normal + sodium chloride	0·981 ± 0·04 (5)*	6·17 ± 0·61 (5)
Normal + thiourea	1·030 ± 0·03 (5)	5·31 ± 0·67 (5)
Thyroidectomized + sodium chloride	1·076 ± 0·07 (6)	3·24 ± 0·19 (6)
Thyroidectomized + thiourea	1·084 ± 0·06 (5)	3·18 ± 0·20 (5)
Thyroidectomized + thiourea + thyroxine	0·939 ± 0·05 (6)	3·22 ± 0·34 (6)

*The figures given in parentheses refer to number of animals employed.

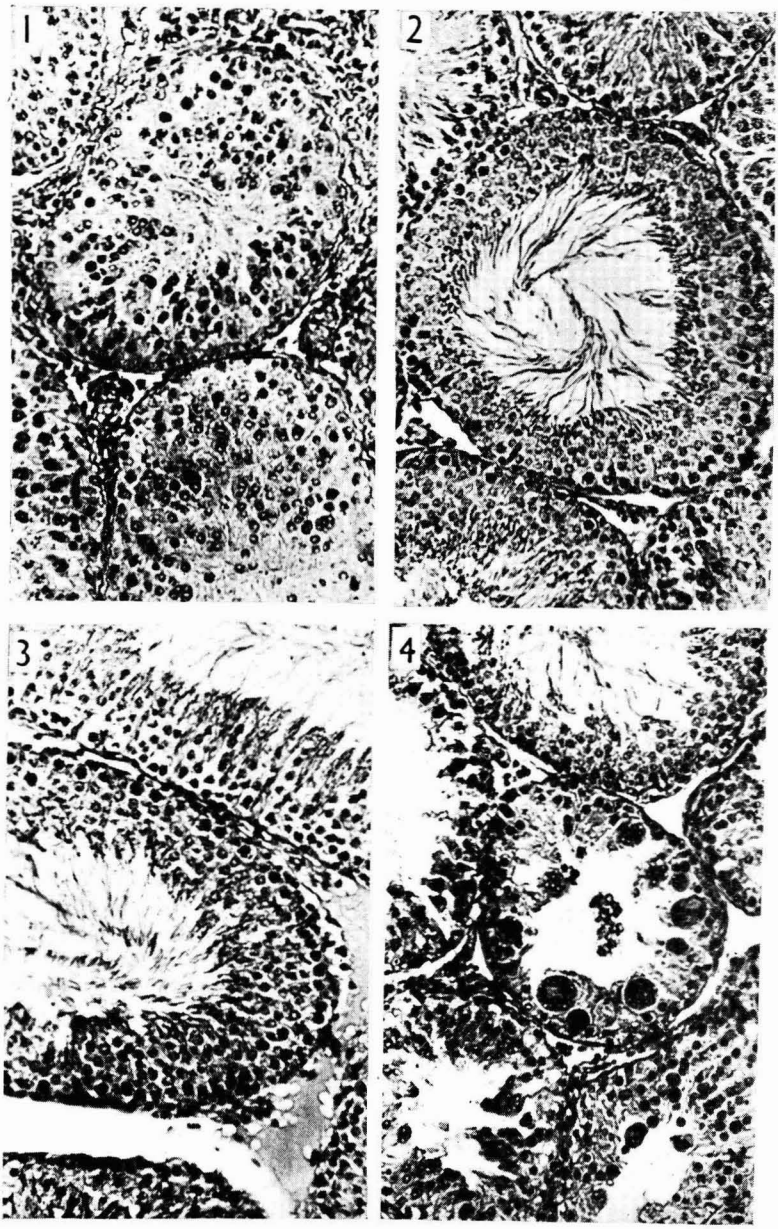

Plate I — Photomicrographs of testis of rats subjected to different treatments × 110 [Fig. 1 : Testis of a rat injected with sodium chloride. Note disorganization of the seminiferous tubules. Interstitium is oedematous but contain healthy Leydig cells. Fig. 2 : Another area from the same testis as in Fig. 1. Note normal tubules and vigorous spermatogenesis. Fig. 3 : Part of the testis of a thyroidectomized rat injected with sodium chloride. Note full spermatogenesis and normal Leydig cells. The interstitium is oedematous. Compare with Figs. 1 and 2. Fig. 4 : Testis of thyroidectomized rat injected with thiourea. Note severe degeneration of the tubules which contain many multinucleated giant cells. The interstitium contains healthy Leydig cells. Compare with Fig. 1]

sodium chloride ($P < 0.01$, Table 1). The histological changes were interesting, too. Thus, in the testis of normal animals injected with sodium chloride focal degeneration was the noteworthy feature. In the tubules mostly the terminal stages of spermatogenesis involving the spermatozoa, spermatids and secondary spermatocytes were affected. There was exfoliation of these elements, hyalinization and nuclear pycnosis (Plate I, Fig. 1). In occasional tubules the epithelium was a mere debris of dead cells. The interstitium was by and large normal with healthy Leydig cells. There was no disintegration of tunica propria except in the totally necrosed tubules. In areas contiguous to such tubules large number of fibroblasts were seen in addition to healthy Leydig cells. These areas were somewhat oedematous and hyperaemic and showed symptoms of inflammatory reactions. The major portion of the organ, however, presented normal features; the tubules were at the height of spermatogenic activity and the histoarchitecture of the interstitium was typical (Plate I, Fig. 2). The histological features of the testis of normal animals given thiourea were more or less similar to those of the normal plus sodium chloride group. However, the degenerative changes were more widespread although their nature was similar. Nevertheless, healthy areas with normal tubules and interstitium were clearly discernible.

In contrast, only mild degenerative changes were seen in the occasional tubules in the thyroidectomized animals injected with sodium chloride. The terminal stages like spermatozoa, spermatids and secondary spermatocytes were affected. The vast majority of tubules were, however, normal and showed full spermatogenesis (Plate I, Fig. 3). Except for a slight oedema, the interstitium presented normal features. Virtually all of the tubules showed mild to severe degeneration in the thyroidectomized animals given thiourea. In most severe cases the gametogenic epithelium was a debris of dead cells. In the majority of the tubules, however, all elements except spermatogonia were degenerated. Giant cell formation was widespread in the tubules (Plate I, Fig. 4). It may be recalled that this feature was virtually absent in the testis of the previous three experimental groups. In tubules with mild degenerative changes only the spermatozoa, spermatids and secondary spermatocytes were affected. In general, extensive sloughing, cytolysis and nuclear pycnosis were the predominant features. The tunica propria was mostly intact. The interstitium was, by and large, normal and the symptoms of inflammatory reactions were minimum. Some normal tubules were, however, seen in the peripheral regions. In thyroidectomized animals injected conjointly with thiourea and thyroxine, the histological features of the testis were more or less similar to the thyroidectomized group treated with thiourea alone.

The results of the present study suggest that the changes evoked by thiourea after direct infusion into the testis are not mediated through the thyroid. Altered osmomolarity due to this method of administration cannot be held solely responsible, either for the observed effects of the compound. This is evident from the nature and magnitude of degenerative changes in the organ after thiourea or equimolar amount of sodium chloride injection. The latter alone causes focal and relatively mild degenerative changes involving mostly the tubules. There is spermatogenic arrest and degeneration of the more terminal elements of the seminiferous epithelium. The relatively mild nature of sodium chloride effect is further indicated by the virtual absence of giant cells in the degenerated tubules. In contrast, the pathological changes are far more widespread and severe in the thiourea-treated animals. This is particularly noticeable in the thyroidectomized animals; except spermatogonia all other elements of the gametogenic series are affected. Giant cell formation is almost universal. Concurrent thyroxine administration is totally ineffective in preventing such degenerative changes. However, in the normal animals the relatively less drastic effect of thiourea on the tubules may be due to the presence of the functioning thyroid; the compound is perhaps removed partially by the thyroid and thereby the severity of testicular change is somewhat reduced. Nevertheless, the bulk of evidence strongly suggests that thiourea is capable of exerting a direct effect on the rat testis.

References

1. Money, W. L., *The Thyroid, Brookhaven Symposia in Biology*, No. 7, New York, **1954**, 137.
2. Kar, A. B. & Das, R. P., *Proc. nat. Inst. Sci., India*, **27** (1961), 56.
3. Selye, H., *Stress* (Acta Inc., Montreal), **1950**, 257.
4. Roy, A. C., Kar, A. B. & Sur, R. N., *Acta Endocr.*, **14** (1953), 254.
5. May, L. G., Moseley, R. W. & Forbes, J. C., *Endocrinology*, **38** (1946), 147.
6. Leathem, J. H. & Seeley, R. D., *Amer. J. Physiol.*, **149** (1947), 561.
7. Dolianiske, J., Eshkol, Z., Givol, D. *et al.*, *J. Endocrin.*, **13** (1956), 131.
8. Karkun, J. N., Roy, S. N. & Roy, S. K., *J. sci. industr. Res.*, **15C** (1956), 246.
9. Maqsood, M., *Biol. Rev.*, **27** (1952), 281.
10. Kar, A. B., Roy, S. K. & Roy, A. C., *J. sci. industr. Res.*, **14C** (1955), 129.
11. Zlatkis, A., Zak, B. & Boyle, A. J., *J. Lab. clin. Invest.*, **44** (1953), 486.
12. Roy, A. C., Dutta, S. N. & Sur, R. N., *J. sci. industr. Res.*, **14C** (1955), 124.

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