DISCUSSION
Microorganisms, in general are endowed with great synthetic ability. Most of them, specially bacteria are capable of growing in simple culture media containing an organic compound, an inorganic nitrogen source and a few inorganic salts. All complex metabolites are built up from these simple ingredients. However, the intracellular control system in bacteria has attained such a high degree of precision that a stringent biochemical economy is automatically clamped, whenever there is a tendency to synthesise a metabolite in excess to what is required by the growing cell. The mechanisms involved in controlling over production of the particular metabolite are known to be operative both at biochemical and genetical levels. In rare instances, organisms may escape such controls and may produce a metabolite, e.g., an amino acid, in great excess and may eventually excrete the product in the surrounding medium.

One method of overcoming the control is the cultivation of auxotrophic mutants on a growth-limiting amount of the
required metabolite. This usually results in accumulation of the precursor of the blocked reaction. The accumulation may under suitable conditions be so high that the phenomenon can be commercially exploited for preparation of the metabolic intermediate concerned. An example of this type is found in case of a lysine auxotroph of E. coli accumulating diaminopimelic acid which has been used for commercial production (Casida, 1956).

A second approach, not involving auxotrophic mutants, is to search out rare, naturally occurring microorganisms which synthesise a particular metabolite in very high concentration and excrete it into the surrounding medium, obviously due to an escape from the controlling forces, - feedback inhibition and repression. Extensive screening programs undertaken in Japan have been rewarded with isolation of a host of organisms which are endowed with high synthetic abilities of different metabolites, specially amino acids, as it will be evident from the literature reviewed earlier.

The present investigation was undertaken with the objective of isolation of microorganisms accumulating lysine. The moderate survey, involving 30 soil samples collected from three districts
of West Bengal, yielded 350 isolates of which 7 were lysine producing cultures, as detected by the bio-autographic technique of Udaka (1960). Ultimately, two cultures, one (CII 19) from among these 7 isolates and another (Ms 5), a chance contaminant, were selected for detailed study, on the basis of production of lysine alone in the culture medium in appreciable quantity. Kinoshita et al. (1957) concluded from extensive screening tests that many kinds of microorganisms produced small quantities of several amino acids and only in rare cases, a single amino acid was accumulated in substantial quantity. In a recent publication Kaleja and Linde (1972) reported that out of a total of 1480 aerobic bacteria isolated by them, 926 produced amino acids. Of these about 12% produced single amino acids, 34% yielded 2 amino acids simultaneously and the rest, a larger number of amino acids. The majority of these bacteria were gram positive bacilli. It may be mentioned here that the two organisms selected and studied in details during the present investigation were also aerobic spore-forming bacteria, belonging to the genus *Bacillus*.
The organisms:

The isolate CII 19 has been identified as a strain of *Bacillus megaterium*. Though it differs in two important biochemical characteristics from the standard description of the type species given in Bergay's Manual, the isolate resembles *B. megaterium* in majority of important characters. The two characteristics in which it differs are Voges Proskauer positivity and ability to reduce nitrate to nitrite. According to the Manual, *B. megaterium* is VP-negative and usually unable to reduce nitrate to nitrite. Since it is well known that such intraspecific variations are not uncommon in bacteria, the isolate may be identified as *B. megaterium*.

The other isolate, Ms 5, has been identified as a variant of *Bacillus coagulans*. It resembles closely *Bacillus coagulans* in most of the characters, including its ability to grow at pH 5.0, but differs from the type species in its inability to grow at 55°C, in having slightly smaller spores and in its inability to hydrolyse starch.
Most of the high yielding lysine organisms belong to the genera *Micrococcus*, *Corynebacterium* and *Brevibacterium* (Table 1). *Micrococcus glutamicus* has later been re-identified with *Corynebacterium glutamicum* (Nakayama and Kinoshita, 1966). Among the *Bacillus* species, *B. subtilis* (Kinoshita, 1959 and Aida et al., 1960) and *B. megaterium* (Sasaki et al., 1965 and Kyowa Fermentation Industries, 1969) are known to produce lysine. The yield of the Sasaki organism was given as 2.89 g per litre. The maximal yield obtained with isolate CII 19 corresponds to 1.85 g per litre under normal conditions, but it increases to 2.35 g per litre in presence of 0.01 μg of erythromycin per ml of growth medium. It is observed, therefore, that the yield of CII 19 compares well with that reported by Sasaki et al. (1965) for their *B. megaterium*. The yield of the other isolate, Ms 5, is about 1.43 g per litre under normal conditions and is about 2.15 g per litre in presence of 0.01 μg of erythromycin per ml of medium. It has already been mentioned that lysine production has been reported from only two species of *Bacillus* (loc. cit.). The isolate Ms 5 which has been identified as a variant of *B. coagulans* may be
taken to constitute the first report of lysine production from this species. It must be admitted, however, that considering the yield of lysine reported from different high-yielding auxotrophic strains of Corynebacterium glutamicum and Brevibacterium (Table 1), the yield by other bacteria, such as Streptomyces, Cellulomonas, Bacillus, Mycobacterium etc. is poor. The yield data of the isolates CII 19 and Ms 5 also conform to this general rule and prove no exception.

Growth and lysine accumulation:

The relationship between growth of an organism and formation of a product can be of two distinct types. In the first type, the product is a direct function of growth, so that the quantity of product is proportional to the amount of growth. In the second type, there is little product formation during active growth phase which is followed by a phase of product formation during which there is little or no active growth (Weinshank and Garver, 1967). In case of amino acid producing microorganisms, usually the first type of relationship is exhibited. The accumulation of amino acid in medium is a
function of total biological mass of the culture. This has been noticed in Micrococcus glutamicus mutants accumulating lysine (Kinoshita et al., 1958) and in Corynebacterium sp. (Plachy, 1970). In the present investigation, the growth-yield curve of B. megaterium, CII 19 (Fig 6) conforms in general to the first type. In case of the other organism (Ms 5), however, a somewhat different type of growth-yield relationship was observed (Fig. 7). About 25% of the accumulated lysine is produced during the period of active cell growth, while the rest of it when there is little active growth. The growth-yield relationship in this organism may be taken to form an intermediate type between the two distinct classes mentioned by Weinshank and Garver (1967).

Carbon and nitrogen sources:

As the quantity of an amino acid accumulated is in the ultimate analysis dependent on the total amount of growth, the growth medium should be so constituted that a maximum crop of cells may be obtained. The most important constituent of a
growth medium in case of heterotrophic organisms is a suitable organic compound which can be dissimilated by the organism to obtain energy and the carbon skeleton of the large number of biopolymers it requires for formation of its body. The next in importance is a suitable nitrogenous compound.

Among 12 different compounds tested as C-source, pentoses were not utilized for growth and lactose and mannose for lysine accumulation by *B. megaterium* (CII19), as evident from Table 8. Although mannose supports comparatively little growth, lactose happens to be a good C-source for growth of this organism. It appears difficult to foresee, therefore, why it fails to support any amino acid accumulation. The fact becomes more perplexing because both galactose and glucose individually support amino acid accumulation by this organism. Among the tested C-sources sucrose, starch and maltose prove to be the best so far as the yield of lysine is concerned.

The other isolate, Ms 5 can not utilise starch and glycerol for growth and again lactose and mannose for lysine accumulation. The best C-sources for amino acid production
are glucose, maltose and sucrose (Table 8). For both the organisms, sucrose was found to be a good C-source and it was used in most of the later experiments. Sucrose has been reported to be a carbon source of choice also for other lysine producing organisms, as Corynebacterium sp. (Plachy, 1970), Ustilago maydis (Sanchez-Marrowuin et al. 1970), M. glutamicus (Welward et al. 1971; Bucko et al. 1971).

The highest yields of lysine under laboratory conditions were obtained with 6% sucrose medium in case of B. megaterium (CII 19) and with 8% sucrose in B. coagulans (Ms 5). The consumption of the sugar during growth was not followed, however. Kinoshita et al. (1958) used 5% glucose for M. glutamicus (613-1) Kyowa Fermentation Industries (1960) used 2.5% glucose for M. glutamicus (ATCC 13032); Plachy (1970) used 10% sucrose for Corynebacterium sp.; Kyowa Fermentation Industries (1971) also used 7.5% glucose for Brevibacterium ammoniagenes; Nakayama and Kase (1971) used only 1% glucose for Corynebacterium glutamicum and Chatterjee (1971) used 9% glucose for Bacillus cereus var. mycoides and 8.5% glucose for B. subtilis. The quantity of sugar consumed and the rate of consumption are dependent to a
large extent on the degree of aeration in case of aerobic bacteria. The results obtained from laboratory experiments can, therefore, be hardly compared with those of fermentors.

Among the nitrogen sources tested, ammonium chloride was found to be the best for lysine accumulation of both B. megaterium (CII 19) and B. coagulans (Ms 5). On the other hand, asparagine was the best N-source for growth of both the organisms. The isolate CII 19 could utilise, among other compounds, urea while the other isolate (Ms 5) could not (Table 11). As for the optimal concentration of nitrogen sources, it was observed that at 0.6 - 0.8 g of ammonium chloride per 100 ml of medium, both growth and lysine accumulation were at best. In most of the reported studies on microbial lysine accumulation, ammonium salts have been used in conjunction with a complex nitrogenous compound, such as corn steep liquor, soybean meal extract, peptone, meat extract etc. (Kinoshita et al., 1958; Ajinomoto Co., 1970, 1971; Mheem and Kwon, 1971; Nakayama and Kase, 1971; Tanaka et al., 1971; Oki et al., 1972; Okumura et al., 1972 etc.). In the present investigation, however, only a synthetic medium was used.
Medium supplements:

As lysine production in a simple mineral medium containing glucose or sucrose as C-source was not much, attempts were made to induce an increased lysine accumulation by supplementation of the mineral medium with yeast extract and casein hydrolysate. Both of these substances have been used widely for other lysine producing organisms (Kinoshita et al., 1958; Kyowa Fermentation Industry, 1960; Seto, 1962; Seto and Harada, 1970; Kyowa Fermentation Industry, 1971; Tanaka et al, 1971; Nakayama and Kase, 1971; Nakayama and Araki, 1971). From the date presented in Table 6, it may be observed that both these supplements singly or jointly stimulate growth of *B. megaterium* (CII 19) to a considerable extent. Lysine accumulation, on the other hand, is strongly inhibited by yeast extract in this organism but not by casein hydrolysate. The latter shows stimulation of lysine accumulation which is proportional to stimulation of growth. Yeast extract exhibits complete inhibition of lysine accumulation at a concentration of 50 mg per 100 ml. An explanation for this inhibition was found later, while the effect of B-vitamins on this organism was studied.
The effect of B-vitamins of the B-group on growth and lysine accumulation of *B. megaterium* (CII 19) was studied. It was observed that the vitamins can be grouped into 3 categories. The first category, including thiamine, pyridoxine and para-amino benzoic acid, stimulate lysine accumulation by 40-48%; growth is not stimulated much, except to small extent in case of p-amino benzoic acid (Table 13). The second category of vitamins, including biotin and pantothenic acid show strong inhibition of lysine accumulation and at the same time, show remarkable stimulation of growth. These effects are much more pronounced with biotin than pantothenic acid. At a concentration of 1 μg per litre biotin completely inhibits lysine accumulation and stimulates a 4-fold increase in growth compared to control. The third category of vitamins, including nicotinic acid, riboflavin and cobalamine, has no effect either on growth or lysine accumulation.

Effect of vitamins on lysine accumulation has been studied by Shigato (1962) in *Micrococcus glutamicus*, by Areshkina et al., (1965a, 1965b) in *Brevibacterium* sp., by Kutseva and Klyueva (1970) also in *Brevibacterium* sp.. Apart from them, B-vitamins
have been used in growth media of many other lysine producing bacteria (Ajinomoto Co., 1970; Kyowa Fermentation Industry, 1971a, 1971b; Adachi and Seto, 1971; Nakayama and Kase, 1971; Okumura et al., 1972; Oki et al., 1972 etc.). Shigato (1962) reported that all lysine producing strains of *M. glutamicus* required biotin. Similarly, Areshkina et al., (1965a, 1965b) found that biotin was essential for optimal lysine accumulation by *Brevibacterium*.

The exact role of biotin in amino acid producing microorganisms is not clear. While Tanaka et al., (1960a, 1960b) hold that biotin functions by limiting growth and allowing carbon and nitrogen to the formation of amino acids rather than to the synthesis of cell matter, others (Shiio et al., 1962; Otsuka et al., 1965; Veldkamp et al., 1963) believe that low biotin concentration makes the bacterial cells more permeable allowing a higher leaching out of amino acids into the surrounding medium. The data obtained in the present investigation support neither of these two hypotheses. To test whether addition of biotin makes the cell wall less permeable to lysine, thus accounting for absence of lysine accumulation, the content
of intracellular free lysine was estimated of cells harvested from biotin rich medium. It was observed that intracellular free lysine content of these cells was even poorer than that of cells grown in absence of biotin. This observation together with a remarkably increased growth at high biotin level seems to suggest that biotin and probably also pantothenic acid lead to a better utilisation of the amino acid for building of cell materials.

The lysine producing strain of *B. megaterium* (CII 19) has been observed to accumulate also $\alpha$-alanine when the medium has a pH value of 7.0 or above (Table 23). It is interesting to note that the concentration of biotin which completely inhibits lysine accumulation does not affect $\alpha$-alanine accumulation. The optimum concentration of biotin for $\alpha$-alanine accumulation was found to be 10 $\mu$g per litre, whereas complete inhibition of lysine occurs at 1 $\mu$g per litre concentration. At 100 $\mu$g per litre concentration of biotin, however, growth and $\alpha$-alanine accumulation both decrease (Table 28). This observation does not fit in with
the permeability theory of Shiio et al. (1962) and others. Because, had the lysine accumulation been stopped at high biotin level due to a change in permeability of the cell wall then \(\alpha\)-alanine excretion should have also stopped. The data rather suggested that biotin induces a change in the metabolic pattern, a change which involves stoppage of over production of lysine without affecting the over production of \(\alpha\)-alanine. A change in metabolic pattern as a function of biotin was first suggested by Kinoshita (1960) with reference to a glutamic acid producing strain of \textit{M. glutamicus}.

The second organism, \textit{Bacillus coagulans} (Ms 5), is nutritionally more complex than the other organism. It has an absolute requirement of methionine and thiamine for growth, but this growth supporting medium does not allow lysine accumulation, for which two other factors, - threonine and pyridoxine are essential (Table 7). The optimal concentrations of thiamine and methionine for growth were estimated as 0.01 and 50 \(\mu\)g per ml of medium respectively. On the other hand, for maximal production of lysine, 0.1 \(\mu\)g per ml of pyridoxine and 50 \(\mu\)g per ml
of threonine are required. At higher concentrations, thiamine and threonine, however, act adversely on lysine accumulation (Tables 16, 17 and 19).

Regarding the effects of other vitamins of the B-group it was noticed that none had any stimulatory or inhibitory influence on lysine accumulation, although growth was stimulated by biotin and to a lesser extent by pantothenic acid (Table 18). It may be noted that the two species of *Bacillus* used in the present investigation differ significantly in their behaviour towards biotin, so far as lysine production is concerned.

Effect of pyridoxine on lysine or diaminopimelic acid accumulating organisms has been studied by Chas. Pfizer & Co. (1959) and Nubel (1961). It was reported that on one hand pyridoxine helps in the conversion of DAPA to lysine and on the other, DAPA accumulation by *E. coli* auxotrophs was markedly increased in presence of the vitamin.

Most of the high-yielding lysine bacteria are auxotrophic mutants, requiring homoserine or threonine and methionine. A
few of these bacteria are also isoleucine auxotrophs. All these amino acids including lysine belong to the aspartic acid family and their biosynthetic pathways are interconnected as shown on the next page (Umbarger and Davis, 1962):
COOH  |  CO - P  |  CH = O  |  COOH
CH2   |  CH2     |  CH2    |  CH2
|       |       | Pyruvic acid       |
|      |       | Succinyl CoA       |
| CHNH2| CHNH2  | CH2    | CH2
|      | COOH   | CH2    | CH2
|      |        | CH2    | CH2
|      |        | H-C- NH- C=O |
|      |        | COOH   |

Aspartic acid  Aspartyl phosphate  \( \beta \)-semialdehyde  Homoserine  Cysteine  Cystathionine  Homocysteine  Methionine  Threonine  L-Lysine  meso-Diaminopimelic acid
During the present investigation the effect of adding methionine and threonine in the growth medium on lysine accumulation was studied (Tables 19 and 20). As it has already been mentioned, methionine is essential for growth and threonine for lysine accumulation of Ms 5. At above-optimal concentrations, methionine does not influence growth or lysine production of Ms 5. But at such concentrations threonine inhibits lysine accumulation but not growth. In case of *B. megaterium* (CII 19), methionine at 50 μg per ml concentration stimulates lysine accumulation only slightly and so also does threonine. At higher concentrations there is inhibition of neither growth nor lysine accumulation. Stimulation of lysine production by threonine and methionine has been observed in other organisms, but these are mostly auxotrophic for these amino acids. Thus Alikhanyan (1966) obtained best production of lysine by *M. glutamicus* using 300 - 400 μg/ml threonine and 300 - 500 μg/ml isoleucine. Daoust and Stoudt (1966) showed that in a homoserine-less auxotroph of *M. glutamicus*, threonine inhibited lysine synthesis to a degree proportional to its concentration in the medium above a critical level. Methionine was slightly
inhibitory only at relatively high levels. Similar observations regarding the inhibitory effect of threonine was also made by Kinoshita and Nakayama (1966). De Zeeuw (1963) too observed that threonine at a concentration of 500 /ug/ ml exerted a 87% inhibition of lysine accumulation. How much of these inhibitions is due to feed-back inhibition is not clear from the data presented by these authors.

Considering the importance of auxotrophic mutants in lysine production, such mutations were induced in B. megaterium (CII 19). Several auxotrophs were obtained requiring isoleucine, proline, serine, glycine, tryptophan and thiamine, all of them having single requirements. But these mutations did not appear to affect lysine yield to any remarkable extent.

Role of pH of growth medium:

An interesting aspect of B. megaterium (CII 19) was revealed through the study of the effect of pH of the growth medium on amino acid accumulation. It was observed earlier in the time course experiment that the pH of the medium falls from 7.0 to 6.0 in course of 60 hours. On testing the effects of
different pH values (Table 23), it was found that lysine accumulation reaches an optimum at pH 6.8. But, when the medium has a pH value of 7.0 and above, a second amino acid, \(\alpha\)-alanine, is accumulated. Above pH 7.4, lysine accumulation stops, but \(\alpha\)-alanine accumulation continues till pH 7.8. Thus this organism is a potential producer of two amino acids. Such an effect of pH in bacteria producing lysine, or any other amino acid has not been reported before. Modifying effects of other external factors on amino acid accumulation, however, are on record. Thus, Nakayama et al. (1960, 1961) observed that a lysine producing strain of \textit{M. glutamicus} produced glutamic acid when homoserine (or threonine plus methionine) and isoleucine were present in excess and when biotin concentration was low in growth medium. Production of glutamic acid instead of lysine by \textit{Brevibacterium} was also reported by Kutseva and Klyueva (1969) when to the growth medium 2 - 4 units of penicillin per ml was added. Kutseva and Klyueva (1970) reported that the same organism produced \(\alpha\)-alanine instead of lysine, when the medium contained less than 100 \(\mu\)g thiamine per litre.

In \textit{B. coagulans} (Ms 5), pH of the medium does not change during growth (Table 22).
Role of antibiotics, gentian violet and Tween 80 on lysine accumulation:

There are some reports regarding increased lysine production in presence of small quantities of several kinds of antibiotics. Thus Shigato (1963) used erythromycin and oleandomycin in *M. glutamicus*. Higher yields were also obtained by Bucko et al. (1971) in an auxotroph of *M. glutamicus* by using mycelial extracts of *Penicillium chrysogenum* and *Streptomyces aureofaciens* from spent fermentations of penicillin and tetracyclain respectively. The exact role played by antibiotics in inducing increase of lysine production is though not clearly understood. So far as penicillin and related antibiotics are concerned, it may be assumed that the cell wall synthesis of the bacteria is affected, resulting in a change in the permeability. Such an effect was observed by Birnbaum and Demain (1969) in glutamic acid excretion by *Corynebacterium glutamicum* in presence of penicillin. They deduced that increased excretion was due to the alteration of the permeability of the cell membrane. On the other hand, Kutseva and Klyueva (1969) claimed that penicillin not only induced a change in permeability, but also brought about a change in the enzyme system participating in
lysine biosynthesis, as they observed that their strain of lysine producing *Brevibacterium* accumulated glutamic acid in presence of penicillin.

During the present investigation the effect of neomycin, bacitracin, erythromycin and gentian violet was studied. Penicillin was excluded because both the organisms were found to be resistant against 100 units of it per ml. The reason for including gentian violet is that it is known to inhibit cell wall biosynthesis in bacteria, just as penicillin, bacitracin, oxamycin etc. Results presented in Tables 24, 25, 26 and 27 show that bacitracin and gentian violet have insignificant improving effect on lysine accumulation. Neomycin at 0.01 μg per ml increases lysine accumulation by about 52% in *B. megaterium* (CII 19) and by 31% in *B. coagulans* (Ms 5). Erythromycin has a somewhat better effect. At 0.01 μg/ml it induces an increase of lysine yield of about 83% in CII 19 and about 48% in Ms 5. At higher concentrations, it inhibits growth and lysine accumulation of both the organisms.

Surface active agents are also known to affect membrane permeability. Watanabe et al. (1965a, 1965b) observed that
conversion of aspartic acid to α-alanine by *Xanthomonas oryzae* was enhanced in presence of sorbitan monopalmitate, sodium lauryl sulphate and sodium oleate. Rehacek and Basappa (1971) found that addition of Tween 80 to a submerged culture of *Claviceps paspali* increased the formation of biopolymers and the phase of maximal alkaloid formation by this fungus. Watanabe et al. (1972) used several surfactants in lysine fermentation by *Arthrobacter alkanicus*.

In the present investigation the effect of Tween 80 was studied. Results given in Tables 26 and 27 show that Tween 80 has a marked influence on cell growth but lysine accumulation is adversely affected. At 0.05% (v/v) concentration it causes about 100% increase of growth over control but lysine accumulation is as in the control or slightly lower. At higher concentrations, growth stimulation and lysine production are more and more retarded and at 0.5% concentration it is totally inhibitory for growth. It is interesting to note that whereas lysine accumulation of *B. megaterium* (CII 19) is unaffected at 0.05% concentration, α-alanine production by the same organism is
enhanced by about 61% over control. At higher concentrations, the inhibitory effect also becomes evident on $\alpha$-alanine accumulation (Table 29).

**Isolation and purity of product:**

Isolation of the excreted lysine from culture broth by ion-exchange chromatography, using the weak cation exchanger Dowex-50 either in the $H^+$-form or in the $Na^+$-form proves no problem. The yield was, however, about 55% of the total quantity present in the broth. Recovery from broth using Dowex-50 in $NH_4^+$-form, however, did not appear suitable. Gordienko et al. (1966) used a cation exchanger in $H^+$-form and reported a recovery of 76%. They also used the exchanger in $Na^+$-form and obtained a recovery of 65 - 78.5%. Dronov et al. (1966a, 1966b), on the other hand, used several types of cation exchangers in $NH^+$-form and got a recovery of about 80%. The failure to recover lysine in a column of resin in $NH_4^+$-form during the present investigation may possibly be due to the fact that the resin used by Dronov et al. are of a kind different from Dowex-50.
Lysine could be recovered from the eluate by concentration and precipitation with ethanol. Its homogeniety was tested by paper chromatography using a number of different developing solvents, in each of which the isolated product formed a single spot, corresponding to that produced by an authentic sample of lysine. That the isolated sample consisted of the L-isomer was proved by growing a lysine requiring auxotroph of *E. coli* on the isolated sample and an authentic sample of lysine in parallel sets and comparing the amounts of growth of the auxotroph in the two sets (Fig. 9).