CHEMICALS

NAD (NADH), nicotinamide adenine dinucleotide oxidised (reduced); NADP (NADPH), nicotinamide adenine dinucleotide phosphate oxidised (reduced); GSH, reduced glutathione; FMN, flavine mononucleotide; ATP, adenosine triphosphate; CoA, coenzyme A; malonyl-CoA; acetyl-CoA; dl-isocitric acid; di-thiothreitol; avidin; biotin; D-mannose; D-methyl mannose; D-glucose; D-methyl glucose; Tris (Sigma 121); L-asparagine; methyl lignocerate and methyl behenate were the products of Sigma Chemical Company, St. Louis, Mo., U.S.A. Fatty acid methyl esters for gas chromatography were obtained from National Institute of Health, Bethesda, Maryland, U.S.A. Tuberclos-tearic acid and methyl hexacosanoate were generous gifts from Prof. E. Lederer (Institut de Chimie des Substances Naturelles, Gifsur-Yvette, France) and acyl carrier protein was kind gift from Prof. P. Lynen (Max Planck Institute, Dortmund, W. Germany).

Inorganic, organic chemicals and solvents used in the present study were of analytical grade and were purchased from British Drug House Ltd., Bombay (India). All the organic
solvents were distilled before use in a glass apparatus.

Nitrogen, hydrogen and oxygen were obtained from Indian Oxygen Co. (Ltd.) and were 99.9 per cent pure.

Radioactive chemicals: Sodium-1$\textsuperscript{14}$C acetate; sodium-2$\textsuperscript{14}$C malonate; palmitic acid-1$\textsuperscript{14}$C; octanic acid-1$\textsuperscript{14}$C; propionic acid-1$\textsuperscript{14}$C and acetic-1$\textsuperscript{14}$C anhydride were obtained from Bhabha Atomic Research Centre, Bombay. Labeled acyl-CoA derivatives along with labeled malonyl-CoA were prepared in the laboratory.

Organisms: Two species of mycobacteria were used in the present investigation. They were Mycobacterium tuberculosis Var hominis strains H$_{37}$Rv and a saprophyte Mycobacterium smegmatis. These cultures were originally obtained from National Collection of Type Cultures, London.

Storage of cultures: M. tuberculosis and M. smegmatis were maintained on Lowenstein–Jensen medium and kept for a suitable period (usually six months). The most satisfactory method is to place the cultures in a deep freeze cabinet at $-20^\circ$C. At this temperature the viability is maintained up to two years. For subculture the slant is removed from the deep freeze and allowed to thaw at room temperature before transferring the growth to a fresh medium.
Lowenstein-Jensen medium: The medium is prepared by dissolving 4 g potassium dihydrogen phosphate, 0.4 g magnesium sulfate, 1 g magnesium citrate, 6 g asparagine, 20 ml glycerol in one litre of water. 20 ml 2% malachite green solution and 600 ml of the above mineral solution were added to 100 ml of egg fluid obtained from 10 washed and alcohol sterilized beaten eggs under sterile conditions. The pH of the medium was adjusted to 7. After mixing, it was allowed to stand for one hour to remove air bubbles. 5 ml of the medium was transferred into each screw capped vial under sterile conditions. The bottles were kept for one hour at 90°C to form smooth slants. Sterility of the medium was tested by incubating at 37°C for one day. The medium was stored at 4 °C.

SELECTION OF THE MEDIUM

*Mycobacterium tuberculosis* H37Rv is an obligate aerobe and grows at 37°C. Surface cultures have been employed in the present study as they are known to give better yields (Goldman, 1961). A variety of media are available (Soltys et al., 1952) for the growth of tubercle bacilli. The synthetic liquid medium by Youmans et al. (1947) has been commonly employed for metabolic studies, where large amounts of cells are required.
Youmans et al. (1947) and Goldman (1956) observed better growth of a virulent strain of *M. tuberculosis* H$_3$7Rv by supplementing the medium with bovine or human serum or plasma, while such an effect was noted by Holmgren et al., (1952) for an avirulent strain (*Mycobacterium tuberculosis* H$_3$7Ra). Since the present study was a comparison of two mycobacterial strains, it was desirable to have identical growth conditions. Hence the medium chosen for the present study had the following composition:

<table>
<thead>
<tr>
<th>Component</th>
<th>gm/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Asparagine</td>
<td>5.0</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>5.9</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20.0</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.5</td>
</tr>
<tr>
<td>Magnesium carbonate</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Each component was dissolved completely then the other was added. The pH of the solution was adjusted to 7.0 with 40 per cent sodium hydroxide. The medium was dispensed in 80 ml quantities in 250 ml conical flasks. For growing the cells in bulk amounts the Haffkine flasks (4 litre capacity) containing one litre medium were used. The medium was sterilized at 15 lbs. pressure for 20 minutes. The sterility of the medium was checked by incubating the
sterile medium at 37°C for 48 hours and the flasks with the turbid medium were rejected.

Inoculum from one day old culture of *M. smegmatis* and from 14 days old culture of *M. tuberculosis H₃₇Rv* was used for subculturing. The cells after 3 days of growth of *M. smegmatis* and 21 days of growth of *M. tuberculosis H₃₇Rv* were harvested by filtration, washed repeatedly with chilled double distilled water and then twice with 0.1 M phosphate (containing 1-mM dithiothreitol-1mM EDTA) buffer, (pH 7.0) to remove traces of medium. The bacterial residue was pressed between folds of filter paper and weighed. From the weighed bacterial mass different portions were taken for the preparation of crude cell-free extracts.

**Preparation of cell-free extracts:** The whole cells were ground in a mortar with 0.1 M phosphate (containing 1-mM DTT-1-mM EDTA) buffer, pH 7.0, to make a homogeneous suspension of 250 mg of cell material per 10 ml. The whole cells suspensions, thus prepared were subjected to sonication in a Raytheon sonic oscillator (KH₂) for 30 minutes at 3-4°C with 5 minute intervals after every 10 minutes. The preparation was centrifuged at 10,000 x g for 30 minutes at 0-4°C in a refrigerated centrifuge (IEC) (Model No. B-20A). The supernatant thus obtained was
subjected to ultracentrifugation at 105,000 x g for 60 minutes in a Beckman preparative ultracentrifuge (model L2-65B). The supernatant obtained was used as the source of the crude fatty acid synthetase.

ASSAY METHODS

A. Assay of incorporation of $^{14}$C acetate into fatty acids by crude cell-free extracts and sonic-extracts.

Fatty acid synthetase and desaturase fatty acid synthetase activity were assayed in a final volume of 1 ml with the following in um: potassium phosphate buffer (pH 7.0) (containing 1-mM EDTA and 1-mM DTT) 100; CoA 0.25; ATP 3; KHCO$_3$ 25; NADPH 1 (or NADP 1 and isocitrate 2.5); NADH 1; reduced glutathione 0.8; MnCl$_2$ 1; sodium-$^{14}$C acetate (0.5 uci) 1 and enzyme protein (2-5 mg). Incubation was carried out in a glass-stoppered tube at 38° for 2 hours. In earlier work, the reaction was stopped by adding 1 ml of 10 per cent (w/v) KOH in ethanol, then saponified for 30 minutes at 80-85° and after acidification with HCl to pH 2.0, was extracted with 3 x 5 ml of light petroleum ether (b.p. 40-60°) (Pierard et al., 1963). Routinely the method of Winder et al., (1964) was followed. 2 ml of 10 per cent KOH in ethanol was used and saponified for 5 hours by refluxing
in boiling water bath. The 'non-saponifiable' lipids were extracted with diethyl ether (3 x 5 ml), the aqueous residue was adjusted with HCl to pH 2.0 and 'saponifiable' lipids were extracted with diethyl ether (3 x 5 ml). Etheral extracts were evaporated to a small volume under a stream of air and transferred quantitatively to scintillation vials and the radioactivity was determined. The results were represented as radioactivity (i.e., counts) mg protein/min., present in the reaction mixture.

B. Assay with partially purified fatty acid synthetases.

ACP-independent system (I): The assay mixture contained,
in a final volume of 0.5 ml, the following (in uM):
2-\textsuperscript{14}C-malonyl-CoA 10 (1.5 uci/uM); acetyl-CoA 5; NADPH 3.5;
NADH 3.5; FMN 0.5; DTT 0.5 (mmole); EDTA 0.5 (mmole);
fraction A 80 ug; and potassium phosphate buffer (pH 7.0), 100. The reaction was initiated by the addition of 40 ug protein. Assay mixture was incubated for 25 minutes at 38°. After the reaction was stopped by the addition of 1 ml of 10 per cent alcoholic KOH, samples were saponified at 100° for 30 minutes. Following acidification to pH 2.0 with HCl, the fatty acids were extracted with petroleum ether (3 x 5 ml), solvent was evaporated, and radioactive residue dissolved in 10 ml of scintillation fluid and counted in a Beckman scintillation counter (LS-233).
C. ACP-dependent system (II): The assay mixture contained in a final volume of 0.5 ml, the following in i, uM:
2-14C-malonyl-CoA 5 (1.5 uci/uM); acetyl-CoA or octanyl-CoA 2.5; NADPH 3.5; NADH 3.5; FMN 0.5; E. coli ACP 45 ug; DTT and EDTA (0.5 mmole each); potassium phosphate buffer (pH 7.0) 100; and 50 ug of enzyme protein. Incubation was for 25 minutes at 38°. Other conditions were the same as for the above A and B.

D. ACP-dependent system (III): The assay mixture of this system was the same as for the System-II except that the substrate primer was palmityl-CoA (2.5 uM).

Protein estimation: In crude cell-free extracts the protein was estimated by turbidity method (Stadtman et al., 1951). With the purified fatty acid synthetase the protein content was measured by optical density at 280 mu using a Beckman DU-spectrophotometer.

Fatty acid synthetase activity: 105,000 x g supernatant was used as the source of fatty acid synthetase and the incorporation of the labeled substrate (1-14C-acetate or 1-14C-acyl-CoA derivative) into long-chain fatty acids was taken as the measure of fatty acid synthetase activity. The
specific activity was expressed as millimicromoles (mu moles of $^{14}C$-substrate incorporated per mg of protein per minute

Radioactivity measurements: The radioactivity of the fatty acids synthesized was determined by liquid scintillation counter (Beckman LS-233). The system most commonly used for estimation of $^{14}C$ in aqueous samples by liquid scintillation counting is the dioxan-based system described by Werbin et al., (1959). The system contained, in one litre dioxane: naphthalein 100 g; 2,5-diphenyloxazole (PPO) 10 g; and 2,2'-p-phenylene-bis-(5' phenyloxazole)(POPOP) 2 g.

THIN-LAYER CHROMATOGRAPHY

This technique is being used either by itself or in conjunction with other methods for the separation of almost all the lipid classes. The method is rapid and presents one of the most effective means of analytical separation.

Application of thin-layer chromatography (TLC) for different types of lipid analysis has been the subject of several reviews by Mangold (1961;1964); Malins (1966); Morris (1964;1966); Kuksis (1966;1967) and Renkonen et al., (1967), and special chapters in a number of books (Marini-Bettolo, 1964; Stahl, 1965; Fanderath, 1966 and Skipski et al., 1969).
Different adsorbents are used for thin-layer chromatography, silica gel is used most frequently for separation of lipids. For the present studies silica gel G containing 13 per cent CaSO₄ as a binder (Silica gel G, Merck) was used. The vertical development of the chromatoplates in closed chamber saturated with the solvent system, is the most common method for the separation of the lipids (Padley, 1964) and it has been employed in the present investigation.

Most of the spray reagents used for paper chromatography can be used for detection in TLC, reagents for general detections are described by Mangold (1961), Wollish et al., (1961); Rollins et al., (1964) and Mangold et al., (1968).

Separation of lipids according to their degree of unsaturation:

Separation of lipids according to number of double bonds present in the lipid molecule is based on general property of unsaturated molecules to form complexes with certain compounds. Silver ions are most commonly used for this purpose. 3-5 per cent impregnation is quite sufficient to achieve good separation. Separation of methyl esters of fatty acids has been described by Blank et al., (1963) and Dunn, (1965).
Separation of lipids according to the chain-length of fatty acids:

The separation of lipids according to the chain-length of their fatty acids can be accomplished by reversed phase partition thin-layer chromatography. In this technique, the stationary phase is hydrophobic and the mobile phase is hydrophilic. Paraffin (free of unsaturated and aromatic compounds) or any purified hydrocarbon or silicone oil is used as a stationary phase. Free fatty acids as well as their methyl esters were separated by Lugay et al., (1964) and Paulose (1966) by these methods.

GAS-LIQUID CHROMATOGRAPHY

Since its introduction in 1952 by James and Martin, gas chromatography has proved to be perhaps one of the most important advances ever made in lipid methodology, especially for the study of fatty acids. The versatility of gas-liquid chromatography is such that with suitable modifications of apparatus and technique, gas chromatography can also be used for the separation and characterization of glycerides (Kuksis, 1967), sterols (Horning et al., 1964), bile acids (Sjovall, 1964), carbohydrates and amino acids (Herb, 1968). Nevertheless the reading of what many would consider "ancient history", the original paper on the subject of gas chromatography by James and Martin (1952),
and later studies of many others can provide valuable insight into the reasons for points of apparatus design and application (Scott, 1966; Oshima et al., 1969; James, 1970; Grant, 1971 and Hsuch-Liang et al., 1972).

**Temperature control**: Column temperature control is usually achieved by an air bath with forced circulation, the temperature being controlled by a proportional temperature controller. Generally heating time to a stable temperature is normally greater than that of the column oven and a temporary boost to maximum power is usually used to approach the equilibrium temperature.

**Columns**: Glass columns are ideal for most gas chromatographic analyses of lipid materials since interaction of sample and column wall is negligible (Arnold et al., 1965). With proper handling the fragility of glass columns is not a problem, and they can be emptied and reused. The nuisance of the glass columns is the lack of flexibility in selecting column length or diameter. When samples are injected, a build up of tarry material on the column is often observed. These observations cannot be made with metal columns and injection ports. Stainless steel columns are universally used and are not inferior to glass even in sensitive lipid applications (Hunter et al., 1965; Arnold
Injection port: As mentioned above, decomposition of materials is usually seen with glass columns and may not be noticed with metal injection ports. These should therefore be cleaned periodically by mechanical means since polymerised materials are quite solvent resistant (Smith et al., 1962). With fatty acid methyl ester samples much of the deposit originate in peroxides of unsaturated fatty acids formed prior to gas chromatography, or in non-saponifiable materials retained from the original lipid on transesterification.

The volume of many injection port is large in proportion to the column and sample size. Since parts of the injection port were cooler than others it became customary to operate the main part of the injection port about 50° hotter than the oven temperature to prevent condensation in the cooler parts.

Sample size: The samples commonly used with modern ionization detector systems are of the order of $10^{-5}g$ dissolved in a few microlitres of very volatile solvent.

Detectors: The thermal conductivity detector made gas chromatography a technique with nearly universal applicability, although its usefulness in the analysis of methyl esters of higher fatty acids is limited by a relatively low sensitivity.
The low sensitivity at room temperature is due in part to the necessity of operating the detector at room temperature identical with or slightly above that of the column. The simplest type of argon ionization detector has been used in fatty acid research. This detector is relatively insensitive to variations in carrier gas flow and its own operating temperature. Operation of this type of detector is uncomplicated, and purified argon is generally satisfactory carrier gas in lipid work.

The flame ionization detector has recently overtaken the argon ionization detector as the principal detector employed in lipid research. This detector is less sensitive than the argon ionization detector, but has a better signal to noise ratio, with the result that practical operating sensitivities are similar. Ions produced by combustion of carbon in a hydrogen-supported flame are the basis of operation of this detector, and carbon atoms with a carbonyl function, as in formic acid, or similar types of compound such as carbon disulfide do not give significant response except under particular operating conditions.

The high concentration of water may alter the response characteristics of the flame for other materials without itself giving an obvious peak (Hill et al., 1965; Foster et al., 1965). In some instances water can be recorded (Perry, 1966) or it may displace material from the column.
giving baseline disturbances not due to water itself.

The flame ionization detector requires a supply of hydrogen and air, which may be furnished from cylinders. The ratios of hydrogen and air supplies, and to some extent the relation to the carrier gas, govern sensitivity, linearity and the relative response among structurally differing compounds.

**Gases:** Nitrogen and helium have been almost universally adopted as the standard gas chromatography carrier gases, except with argon ionization detectors. The standard of helium purity has been that of the U.S. Bureau of Mines, and oxygen is stated to be present to the extent of 5 ppm. Argon as used in argon ionization detectors is usually of the same order of purity, but commercially available nitrogen can have up to 100 ppm oxygen unless particular prepurified grades are specified.

**Quantitation:** In practice deviations from ideality may be due to one or more of the following factors: nature of the sample, injection technique, thermal losses on the column, and finally detector load effects or recorder malfunction. Under these circumstances it is highly desirable to calibrate the entire system with the materials being determined under the particular operating conditions employed and over the range of sample sizes anticipated (Vandenheuvel et al., 1965).
Packed columns

Supports: The diatomaceous earth supports used in the gas chromatography of methyl esters of fatty acids are broadly classified as pink or white. The pink supports owe their colour to iron compounds, but the white supports contain about the same amounts of iron. The pink and white supports have considerably different densities and surface areas. Columns prepared from the two types with the same amount of the same liquid phase may have quite different properties. The pink supports generally give higher column efficiencies with non-polar materials, but the surface of the pink supports shows greater surface activity towards polar compounds. The white supports have been prepared for lipid use, particularly the sterols and other difficult compounds (Horning et al., 1963), but even so they often require deactivation by washing with acid and/or alkali by a silanization.

One drawback of the white support has been their greater fragility resulting in fragmentation with loss of column efficiency during coating and packing. This problem has been reduced with the introduction of new supports such as chromosorb G or Gas-chrom Q or W which are more rugged and of lower polarity. All column packing procedures should involve only gentle tapping of the column (Vandenheuvel et al., 1965), but the process should continue until the
column is firmly packed. A gradually increasing vacuum applied at the exit end of the column will assist firm packing. The column should always be used with carrier gas passing in the same end used for packing.

**Liquid phases:** In working with esters of fatty acids the distinction of liquid phases by polarity is based on the appearance of unsaturated fatty acid esters after the corresponding saturated material on polar columns. Conversely, on non-polar columns the unsaturated material emerge first. The classic introductory work on gas chromatography depended heavily on DC 550 silicone oil and Apiezon greases. The latter is still popular and useful in fatty acid research (James et al., 1956).

The polar liquid phases included polyesters prepared from diacids and dialcohol of which ethylene glycol adipate (EGA) (James, 1958) were among the first. More polar polyesters are usually based on succinic acid and butanediol (BDS), ethyl glycol (EGS) or diethylene glycol (DEGS).

**Operating conditions:** The polarity of a polyester phase in respect to analysis of common fatty acid ester mixtures is governed by the type of support, the load of liquid phase, and the operating temperature (Hornstein et al., 1961). Moreover, this polarity usually changes with age (Imaichi et al., 1963), probably owing to general bleeding of the
liquid phase, although selective decomposition may play a role. Non-polar liquid phases may also show some change in polarity, usually one of increasing polarity due to oxidation and/or the retention of polar materials in the column. Most polyesters are stable up to 190-300°, and for best results should not be operated above 220°. At very low temperature the higher boiling materials will give skewed peaks, temperature-programming overcomes this difficulty.

**Standards:** Standard fatty acid methyl esters were obtained from National Institute of Health, Bethesda, Maryland, U.S.A. and were used for calibrating and checking the column and linearity of the detector response.

**Sample application:** The sample was concentrated to a dried volume under nitrogen and injected with a one microlitre syringe.

**Calculation of peaks:** The area of the peak was calculated by multiplication of peak height by width of half height or multiplication of peak height by one half base width. Overlapping peaks were determined by drawing lines tangent to the sides of the peaks and intersecting the baseline.
EXPERIMENTAL DETAILS

Preparation of 1-\(^{14}\)C-acetate solution: Sodium-1-\(^{14}\)C acetate (500 uCi) with a specific activity as 46.8 mCi/mM was dissolved in 10 ml double distilled water. 9.9 umoles of cold acetate was added to 0.1 ml (5 uCi/0.1 ml) so that 1 ml will contain 5 uCi and 0.1 ml of this solution (containing 0.5 uCi/mM) was routinely used in the assay system.

Synthesis of labeled 1-\(^{14}\)C-acetyl-CoA: 1-\(^{14}\)C-acetyl-CoA was prepared from labeled acetic anhydride. 1-\(^{14}\)C-acetic anhydride with a specific activity of 10 mCi/m mole was supplied in 0.1 ml benzene solution; the solution was further diluted to 10 ml with benzene. 0.1 ml containing 5 uCi of the above solution was diluted to 0.5 ml with distilled water and thoroughly mixed. To 0.1 ml (1 uCi) of this solution 13-14 umoles of (0.13 - 0.14 ml) of 0.1 M cold acetic anhydride was added. Benzene was evaporated completely and the material was dissolved in 0.13 - 0.14 ml of distilled water giving 1 uCi/13 - 14 umoles/0.13 - 0.14 ml of acetic anhydride.

The labeled acetyl-CoA was prepared by the method
described by Simon et al., (1933). 10 umoles of coenzyme A (on the basis of free-SH groups as determined by quantitative analysis) is dissolved in 1 ml of water. The solution is cooled to 0°C; 0.2 ml of 1M KHCO₃ is added and the solution is neutralized to pH 7.5. Then 0.13 to 0.15 ml of 0.1 M labeled acetic anhydride is added and after mixing, the solution is allowed to stand at 0°C for 4-5 minutes. If a drop of reaction mixture gives a positive sulphydryl with the nitroprusside solution, more acetic anhydride solution is added until the free sulphydryl is negative. The pH is finally adjusted to 6 with HCl. The labeled 1−¹⁴C-acetyl-CoA, thus produced was 0.15 uci/0.1 umole. Freshly prepared acetyl-CoA was used everyday.

**Synthesis of propionyl-, octanyl- and palmityl-CoA derivatives:** Labeled propionic acid solution supplied had a specific activity of 50 mci/mmole. It was diluted to 10 ml with alcohol, a solution containing 10 uci/0.2 umol/ml, of which 0.1 ml (containing 1 uci/0.02 umole) was taken and 0.98 (0.1 ml) umole of cold propionic acid was added to give 1 uci/umole/0.2 ml. Other solutions were also prepared in the same manner.

Acyl-CoA derivatives were synthesized from the mixed anhydrides of the fatty acids by a modification of the method of thiol ester synthesis as adopted by Goldman et al., (1961).
To 0.6 ml of dry methylene chloride at room temperature were added 14 umoles of 2,4,6-trimethylpyridine and 14 umole of labeled fatty acids. The solution was allowed to stand 10 minutes and then 14 umoles of ethyl chloro-formate were added in 0.2 ml of methylene chloride. After 1 hour at room temperature the mixture was concentrated to dryness on a rotary evaporator and 0.8 ml of freshly distilled tetrahydrofuran were added to dissolve the mixed anhydrides. Under nitrogen 10 mg of CoA (6.5 umole of reduced CoA) were dissolved in 0.8 ml water and the solution was carefully adjusted to pH 8.0 with 1N NaOH. The tetrahydrofuran solution of the mixed anhydride was added and the pH kept at 8 by further additions of 1N NaOH. After 15 minutes the solution was acidified to pH 5.0 with 1 per cent perchloric acid and most of the tetrahydrofuran was removed on a rotary evaporator. The predominantly aqueous solution (approximately 5 ml) was then acidified with 1 ml of 10 per cent perchloric acid and the precipitate was collected by centrifugation at 10,000 x g for 10 minutes. The precipitate was dissolved in 3-4 ml of distilled water and extracted with ether repeatedly until the radioactivity in the ether washes were negligible. Ether was then removed from the acyl-CoA solution under a jet of nitrogen. Solution was adjusted to a concentration of 10 umoles of hydroxamate forming esters per ml. This represented 5.1 umole of enzymatically active CoA
thiol ester and this latter figure was used as the basis for calibrating the acyl-CoA used in the present investigation.

**Synthesis of malonyl-CoA:** The malonyl-CoA is synthesized by the well known method of Trams et al. (1960): 1 mmole of $^{14}$C-malonic acid (specific activity 0.425 mci/mmmole) and 1 mmole of thiophenol were dissolved in 5 ml of N,N'-dimethylformamide at 0°C. To the mixture was added, with stirring over a period of 1 hour, 500 mg of dicyclohexyl-carbodiimide (DCC) in 5 ml of N,N'-dimethylformamide. The mixture was stirred for three hours at 0°C. After the addition of 10 ml of water, the stirring was continued for 15 minutes. The mixture was filtered with suction and the precipitate washed with water. The filtrate was made slightly acidic and extracted with several volumes of ether. The ether was washed with 0.01M HCl and water. After drying over anhydrous sodium sulfate, the solution was shaken out with activated charcoal andfiltered. The purity of thiophenylmalonic ester thus formed was 98 percent as determined by measuring the extinction at 237 μm in ethanol and the yield of hydroxamic acid. The thiophenylmalonate was concentrated under reduced pressure and a slight excess was added slowly with shaking to a solution of 10 umoles coenzyme A in 0.1M bicarbonate buffer at pH
Nitrogen was bubbled through the mixture at 0°C for 3 hours. The mixture was then acidified and extracted several times with ether. In aqueous phase 7.5 umoles of malonyl-CoA was obtained which had an hydroxamate: adenine ratio of 0.66. The aqueous phase was then lyophilized or frozen.

**Purification of malonyl-CoA:** The malonyl-CoA were purified by the method of Ryder *et al.*, (1967). An aqueous solution of malonyl-CoA (20-25 umoles) was applied to a DEAE-50 cellulose column (18 x 1 cms) at -4°C which had been previously equilibrated with a linear gradient of lithium chloride (0.05M - 0.15M HCl). Absorption at 260 nm was measured on each 10 ml fraction and the peak of malonyl-CoA (eluted at about 0.1-0.12 M Lithium chloride) collected. After lyophilization, the malonyl-CoA was dissolved in methanol-acetone (1:4 v/v) and on stirring overnight at -20°C the malonyl-CoA precipitated out. After centrifugation the precipitate (17 mg) was dissolved in water (pH 6.5), stored at -4°C and used within two days of purification. The recovery of thioester during purification was 80-85 per cent. The 2-14C-malonyl-CoA thus produced had a radioactivity as 0.2 uci/1 uM.

**Method of reduction of acyl carrier protein:** The acyl carrier protein (*E. coli*), supplied by Prof. Lynen was
reduced according to the procedure described by him. 10 mg acyl carrier protein (mol. wt. 8,850) approximately 98 percent pure was dissolved in 1 ml of distilled water (1.1 mM solution), 20 μl of acyl carrier protein solution, 1 μl of 1 M tris-HCl buffer, pH 8.5 and 1 μl of 1 M dithiothreitol was incubated for 15 minutes at 37°C. Then 1 μl of 1 M phosphate buffer, pH 7.0 was added. This solution (containing 45 μg of ACP) routinely used for the demonstration of acyl carrier protein requirement.

Preparation of antitubercular drugs solutions: Seven antitubercular drugs which were used in the present investigation were streptomycin sulfate, isoniazid, rifampicin, cycloserine, p-aminosalicylic acid (PAS), ethambutol and morphazinamide. Working solutions were prepared by diluting the stock solutions (1M) with glass distilled water. The rifampicin solution was prepared according to the method of Stottemeier et al., (1969). Stock solution of morphazinamide was prepared in 35 per cent alcohol, alcohol was evaporated by incubating the drug at 38°C for 5 minutes before enzyme was added. Rest of the antitubercular drugs solutions were prepared in glass distilled water.

Using following sets of experiments aliquotes were taken at various time intervals: The antitubercular drugs were incubated with the assay mixture for 5 minutes at 38°C
and then the reaction was started by adding enzyme. Incorporation of substrate in the presence of these drugs was studied at various time intervals.

In another set of experiments 50 umoles of isoniazid were preincubated with enzyme for different time intervals and $^{14}$C-incorporation was studied.

In the third set, the reversal of isoniazid inhibition was studied by the antagonizing substance, pyridoxal phosphate. Using different concentrations of this vitamin and taking aliquotes after incubation, the enzyme activity was determined.

**Preparation of avidin and biotin solutions:** Two concentrations each of avidin (50 ug; 1 mg) and biotin (20 mg; 50 mg) were used. The enzyme was incubated with avidin and biotin along with the assay mixture at 38° for 20 minutes and reaction was started by adding substrate. The results were noted.

**Sulfhydryl inhibitors:** Three sulfhydryl inhibitors, the iodoacetate, N-ethylmaleimide and p-chloromercuribenzoate were studied. Stock solutions were prepared in glass distilled water. The inhibitors (0.1 uM-2.40 uM) were pre-incubated with assay mixture at 38° for 5 minutes, then the enzyme was added and again incubated for
another 15 minutes, aliquotes were taken to determine incorporation of substrates into fatty acids.

Preparation of silver nitrate impregnated silica gel thin layer plates: 4 g of AgNO₃ was dissolved in 10 ml of acetonitrile and then 10 ml of methanol and 80 ml of chloroform were added; 45 g of silica gel G was weighed and shaken up with above medium (4 per cent silver nitrate). Clean, dried glass plates (20 x 20 cms) were coated with the above slurry. The plates were activated in the oven at 110° for 90 minutes and kept in dark. They were always activated before use.

Reverse-phase silica gel impregnated TLC: Glass plates (20 x 20 cms) were coated with silica gel G (E. Merck) to 350 μ thickness according to the method of Mangold (1961). The chromatoplates were activated at 110° for 90 minutes, cooled and were placed in a chamber saturated with 5 per cent liquid paraffin in petroleum ether (40-60°) and allowed to run. This may the plates got coated with liquid paraffin. The plates were air dried and used. The solvent system used was: acetonitrile: acetic acid: water (80:20:10) or 90 per cent acetic acid. The plates were dried to remove the vapours of acetonitrile and acetic acid. Later plates were sprayed with suitable solvent.
Liquid paraffin and silver nitrate impregnated TLC:

This technique was devised by Paulose (1966) for the separation of fatty acid methyl esters based on chain-length as well as unsaturation. The plates coated with liquid paraffin prepared as above, were sprayed with 10 per cent solution of AgNO₃ in 50 per cent methanol. The plates were then developed in 95 per cent aqueous methanol saturated with paraffin and AgNO₃ (saturation was done overnight). The plates were developed for two hours and sprayed with 10 per cent ethanolic solution of phosphomolybdic acid and heated at 200° in an oven for 1 hour, blue spots appeared on a yellow background. The type of separation was not very much different from plates by reverse-phase technique.

DETECTION OF FATTY ACID ESTERS ON THE CHROMATOGRAMS

Iodine: The dried chromatoplates were exposed to iodine vapours for a short period. The methyl esters of unsaturated fatty acids appeared as brown spots on pale yellow background. However, completely saturated lipids can hardly be detected by this method (Mangold et al., 1960; Mangold, 1961; Sims et al., 1962).

Sulfuric acid: The chromatoplates were sprayed lightly with 50 per cent sulfuric acid (v/v) followed by heating in an
oven at 180-200° for 1 hour. All lipids form dark-brown or black spots on the white background. Saturated lipids usually give less intense spots than unsaturated lipids. The former are more resistant to oxidation, and therefore more evaporation can occur before their oxidation to carbon.

**2-7 Dichlorofluorescein:** Non-polar lipids saturated and unsaturated, become visible in ultra-violet light (maximum emission at 270 mu) after the chromatogram has been sprayed with a solution of 0.2 per cent of 2'7 dichlorofluorescein (Eastman Kodak Company) in 95 per cent ethanol. Lipid spots appear as yellow-green on a purple background (Mangold et al., 1960; Mangold, 1961).

**Elution of methyl esters of fatty acids from thin-layer chromatoplates:** After identifying the fatty acid spots and by comparing their Rf values with authentic standards, the bands were scraped into test tubes. To this, 10 ml of n-hexane-ether (1:1 v/v) was added and stirred well with a glass rod at room temperature. The solution was centrifuged for 10 minutes at 2000 r.p.m. and after decanting the solvent carefully, the elution was repeated twice more. The combined solvents were evaporated off and radioactivity was determined by dissolving in scintillation fluid, in a scintillation counter.

**Methylation of free fatty acids:** The commonly used methyla-
tion procedures are: (i) methylation with diazomethane (Schlenk et al., 1960), (ii) methanol in the presence of boron trifluoride (Metcalfe et al., 1961), (iii) methanol and concentrated sulfuric acid (Feldman et al., 1965), and (iv) 2,2'-dimethoxy propane in acid methanol (Abramson et al., 1965).

In the present investigation methylation of total fatty acids samples was carried out according to the procedure of Feldman et al., (1965). The dried ether extracts were transferred to a thick-glass ampoule. 2 ml of 6 percent (v/v) methanolic sulfuric acid were added and the ampoule was sealed in a nitrogen atmosphere. The ampoules were kept sealed in an oven at 80° for 18 hours. They were then cooled and 1 ml of water was added to each ampoule. The methyl esters were extracted with about 5 ml of hexane, added in 1 ml aliquots. The combined hexane extracts were washed once with 5 ml of distilled water to remove traces of acid. The washed hexane layer was passed through a bed of anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen.

**Bromination of methyl esters of fatty acids:** Since it was observed by Lennarz et al., (1962-a) and Subramanyam (1965) that oleate and tuberculostearate had the same retention time on polyethylene glycol adipate columns:
It was essential to brominate the fatty acid methyl esters to measure the percentage of unsaturated fatty acids in the total fatty acid samples. The brominated methyl esters do not separate under these conditions and by comparing the chromatogram of an unbrominated mixture of esters with that after bromination it is possible to deduce which components of the original mixture are unsaturated and to measure accurately the saturated components.

The bromination procedure followed was that of James as modified by Farquhar et al., (1959) is as follows: A small amount of methyl esters (0.5 to 5 ul) was dissolved in diethyl ether in a conical centrifuge tube. This tube was chilled to -10° or lower in a beaker containing dry ice and 95 per cent ethanol. A 22 per cent solution of liquid bromine in diethyl ether was added dropwise to the methyl esters solution, the yellow colour of the solution disappearing as double bonds are brominated. The reaction was carried out to completion as shown by the persistence of yellow colour. Excess bromine and solvent were then completely evaporated under a stream of nitrogen at a temperature less than 30°, for the brominated fatty acid esters tending to crystallise and settle to bottom of the tube. A sample of the supernatant saturated methyl esters was applied to the column with a micropipette.
The conditions for the analysis of fatty acids by G.L.C.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas chromatograph</td>
<td>Perkin-Elmer-990</td>
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<tr>
<td>Column material</td>
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<td>Column outside diameter</td>
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<td>Loading</td>
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<td>Carrier gas</td>
<td>Nitrogen, with 40 ml/mt flow rate</td>
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<tr>
<td>Detector</td>
<td>Flame ionization</td>
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<tr>
<td>Hydrogen and air</td>
<td>Each with 40 ml/mt flow rate having 20 lbs. inlet pressure</td>
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<td>Column temperature</td>
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<tr>
<td>Chart speed</td>
<td>5 min. per inch.</td>
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</tbody>
</table>

Analysis of products of fatty acid synthesis: To determine whether the products of fatty acid biosynthesis were free fatty acids, ACP-derivatives or CoA-derivates, reaction was scaled up 5-fold and run in duplicate for 30 min. (Goldberg et al., 1972). One set of samples was extracted before and after hydrolysis with KOH in order to determine the amount of bound and free fatty acids. The other set
of reaction mixtures was layered on a Sephadex G-75 column (38-ml bed volume). Elution was carried out with 0.15 M potassium phosphate buffer, pH 7.0, and 0.5 ml aliquotes from each fraction (1.5 ml) were counted in liquid scintillation counter. Fractions 12 to 16 (exclusion volume) contained counts associated with protein. Fractions 17-26 contained ACP-bound counts and fractions 27 to 40 contained free fatty acids and labeled-CoA derivatives as was shown by separate chromatography of $1^{14}$C-palmityl-CoA. Fractions were pooled, hydrolysed with KOH, and after acidification they were extracted with three 10 ml portions of petroleum-ether. Solvent was evaporated at 60° and the radioactive residue was either counted for radioactivity or was methylated for separation and analysis by gas-liquid chromatography.

Preparation of stimulating factor: Crude stimulating factor (S.F.) was either prepared as described by Brindley et al., (1969) or more conveniently by the method described by Ilton et al., (1971). A suspension of 100 g of water washed, $M$. $tuberculosis$ H$_{37}$Rv or $M$. $smegmatis$, cells in 500 ml of distilled water was boiled with stirring for 30 minutes. After cooling, the supernatant was separated from the coagulated cells by centrifugation at 8000 r.p.m. for 15 minutes. The extract was concentrated by lyophilisa-
tion to a small volume and an equal volume of acetone was added. The resulting precipitate was removed by filtration and was discarded. Acetone was added to the filtrate to the final concentration of 90 per cent and the precipitated material was collected by centrifugation and dried by washing with ether.

Acid treatment of crude S.F.: Crude S.F. was dissolved in distilled water, pH was adjusted to 2 with acetic acid or 3N HCl. The solution was kept for 30 minutes and the pH was adjusted to 7 with 40 per cent sodium hydroxide. The solution was concentrated and the activity was determined.

Hydrolysis of fractions A and B: Fractions A and B (50 mg each) were hydrolysed with 6N HCl for 24 hours and hydrolysate was tested for carbohydrates, amino-sugars (Elson et al., 1933) amino acids or other ninhydrine-positive materials.

Determination of total sugars by anthrone reagent: Total carbohydrates were determined by an anthrone method (Seifter et al., 1950). Usually, glucose was used as standard and the results are given in glucose equivalents ("Glucose").
**Fluorimetric assay of flavin-mononucleotide:** FMN content of fraction B determined by fluorimeter method described by Burch (1957), using riboflavine standard.

**Paper chromatography:** The carbohydrate constituents of fraction A eluted from ion-exchange columns were hydrolysed and identified by paper chromatography using following solvent systems: (i) n-butanol:pyridine:water (10:3:3); and ethyl acetate:pyridine:water (10:4:3); and (iii) n-butanol saturated with water (iv) n-butanol:ethanol:water (upper phase) 4:1:5. The paper was dried and sugars were detected by either of the following spray reagents: (i) Aniline phthalate, prepared by dissolving 0.9 g aniline and 1.66 g of phthalic acid in 100 ml of n-butanol (Partridge, 1949). After spraying the chromatogram was heated for 5 minutes at 105° to develop the colour. The aldohexoses gave brown spots, while pentoses gave purple colour spots, (ii) Alkali silver nitrate reagent (Trevelyan's reagents, 1950) was prepared by adding 0.1 ml of 50 per cent AgNO₃ saturated solution of 25 ml of acetone. Paper was dried and dipped in 0.5N NaOH in 45 per cent ethanol. After brown AgO spots appeared, the paper was dipped in 1 per cent aqueous sodium thiosulfate to remove dark background and then washed with water.
Acetylation of fraction A: 10 mg fraction A was shaken with a mixture of acetic acid (2 ml), acetic anhydride (2 ml) and 98 per cent H$_2$SO$_4$ (0.25 ml) at room temperature. After 15 minutes a clear solution was obtained which was allowed to stand at room temperature for 40 hours. The acetolysate, slightly brown in colour, was slowly poured into 40 ml of ice water. The mixture was slowly warmed to room temperature and was adjusted to pH 7.5 with dil. NaOH. The suspension of sugar acetate was extracted repeatedly with chloroform until carbohydrate was no longer extractable. The extracts were combined and dried over anhydrous sodium sulfate and evaporated to yield 11.7 mg sugar acetate ('glucose equivalent'). The activity of sugar acetate was determined.

Estimation of fatty acid ester groups in sub-fractions of fraction A: The method of Lee (1966) was used in the present investigation for estimating fatty acid ester groups. The method is based on the ability of carboxylic acid esters to react with hydroxylamine hydrochloride in alkaline solution to yield hydroxamic acid which produces red to violet colour with ferric chloride. The procedure was as follows: To one ml of carbohydrate aqueous solution were added 0.2 ml of 2M hydroxylamine hydrochloride solution and 0.2 ml of 3.5 N NaOH. After 20 min. at room temperature 0.2 ml of 4 N HCl and 0.2 ml of 0.37 N ferric chloride in 0.1 N HCl were added.
added to the solution. Absorbance was measured at 520 nm in Beckman model DU-spectrophotometer. Under these conditions 1 umole of ethyl acetate in aqueous solution yielded 0.28 absorbance units.