On the basis of this similarity in the two systems it would seem to be a justifiable inference that plasma albumin in human blood is the active component.

n should, according to theory, be independent of temperature, as also, the results indicate, may be the case.

SUMMARY

1. 4,4′-Diaminodiphenyl sulphone forms complexes with bovine serum albumin.

2. From a comparative study of these and of the complexes formed in human blood plasma (see preceding paper), it is inferred that, in human blood plasma, plasma albumin is the active component.

3. The data are compatible with the existence of three identical and independent binding sites ($K_1:K_2:K_3 = 9:3:1$), available to 4,4′-diaminodiphenyl sulphone on each albumin molecule.

4. The first complexity constant for the sulphone and human plasma albumin at 37° is $2{\cdot}4 \times 10^3$ l./mole; for bovine serum albumin at 25°, it is $6{\cdot}0 \times 10^3$ l./mole and at 37°, $4{\cdot}5 \times 10^3$ l./mole.

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Studies on the 'Incorporation Factor' with *Bacillus megaterium*

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Gale & Folkes (1958) showed that ultrasonically disrupted cells of *Staphylococcus aureus* (strain Duncan) lost much of their ability to incorporate amino acids into trichloroacetic acid-precipitable material after incubation with deoxyribonuclease. The addition of nucleic acid isolated from the same organism or from *Bacillus megaterium* restored this ability. They further demonstrated that a trace component, which was purified 1000-fold from nucleic acid digests, was capable of replacing nucleic acid. This material, called 'incorporation factor', has also been reported to stimulate the rate of mitosis of chicken fibroblasts (Gale, 1959).

Our initial attempts to investigate the structure of this potent material were hampered by the difficulties of the assay with disrupted *S. aureus* cells [see Gale & Folkes (1958)]. We discovered that the incorporation of [^{14}C]glycine into the trichloroacetic acid-precipitable material of washed and starved intact *B. megaterium* cells was stimulated by an incorporation-factor preparation. We have thus developed a simple and more reproducible assay which has properties similar to the disrupted staphylococcal system of Gale & Folkes (1958). With this assay, a 5-month-old sample of incorporation factor was shown to consist mainly of glycerol by Kuehl, Demain & Rickes (1960). Since the properties of free glycerol do not correlate with some of the characteristics of incorporation factor as described by Gale (1959), Kuehl *et al.* (1960) mentioned the possibility that, in nucleic acid, the incorporation factor might exist as a bound form of glycerol with high specific activity. This possibility is now being studied by E. F. Gale (personal communication). This paper presents the details of our work with the *B. megaterium* system.

METHODS

Organism. B. megaterium was kindly supplied by Dr E. F. Gale of Cambridge University. The culture was maintained by serial transfer on nutrient agar (Difco) slants which were stored at 4°.

Growth and preparation of suspensions. The medium used for growth was similar to the 'deficient medium B' of Gale (1951) and contained 1% of glucose, 0·1% of yeast extract (Difco) and 0·12% of L-arginine hydrochloride in buffered salts mixture. The salts mixture contained 0·1% of KH_2PO_4, 0·33% of Na_2HPO_4, 0·07% of $MgSO_4,7H_2O$ and 0·1% of

NaCl, adjusted to pH 6·25. A 250 ml. Erlenmeyer flask, containing 100 ml. of medium, was inoculated with 0·05 ml. of a suspension of the growth from one slant in 1 ml. of medium. The flask was incubated for 16 hr. at 37° on a shaking machine imparting a rotary motion of 220 rev./min. The culture was well into the stationary phase of growth at this time. From this point on, sterile techniques were discontinued. The culture was centrifuged for 10 min. at 4000*g*; the cells were washed with 20 ml. of buffered salts mixture and then starved (see Results section) at a concentration of 0·7–1·0 mg. (dry weight)/ml. in buffered salts in a water bath for 1 hr. at 37°. After centrifuging, the cells were diluted in buffered salts to about 2·5 mg./ml. and used for incorporation. (Although many experiments were conducted at a lower cell concentration, this was found to be best.)

Incubation. Incubation was conducted in 15 ml. tapered glass centrifuge tubes. To each tube was added 53 μm-moles of uniformly labelled [^{14}C]glycine (0·066 μc), 2 ml. of the buffered salts mixture without NaCl, 1·4 ml. of water or other addition and 0·5 ml. of cell suspension (total vol. 3·9 ml.). The labelled glycine was prepared in the buffered salts mixture without NaCl at the required concentration and kept frozen until used. The NaCl concentration was kept low during incorporation because some of the solutions added contained NaCl after neutralization, and this would have raised the salt content to toxic concentrations if added to the complete salts mixture. The tubes were closed with rubber stoppers and placed in a water bath for 90 or 120 min. at 37°. The concentration of labelled glycine used was apparently sufficient since no diminution in the rate of glycine incorporation was observed throughout the experimental period.

Measurement of radioactivity. The procedure used for measuring radioactivity was modified from that of Gale & Folkes (1953). After incubation, cold 10% (w/v) trichloroacetic acid (4 ml.) was added to each tube followed by 5 mg. of RNA (Eastman Organic Chemicals Department, Rochester, N.Y.). This carrier RNA provided a large trichloroacetic acid-insoluble pellet, preventing loss of incorporated radioactivity during the subsequent washings. To those tubes that already contained nucleic acid from the incubation, enough carrier RNA was added to bring the total to 5 mg. The tubes were refrigerated for 30 min. and centrifuged at 1000*g* for 15 min. The precipitates were washed with cold 5% (w/v) trichloroacetic acid (3 ml.), cold 5% (v/v) acetic acid (3 ml.) and finally with cold 1% acetic acid (2 ml.). Next, each pellet was suspended in 0·75 ml. of aq. 20% (v/v) acetone and 0·3 ml. was spread on each of two aluminium planchets (8 cm.^{2} surface area) containing a drop of 1% polyvinyl alcohol. The planchets were dried *in vacuo* over P_2O_5 and the ^{14}C content was determined with a Nuclear-Chicago Corp. automatic-flow gas counter with a micromil window. In this machine, the 0·066 μc placed in each tube gave 21 000 counts/min. Because of the small amount of solids placed on each planchet, self-absorption was negligible.

Suspension densities. Suspension densities were determined from a calibration curve that related extinction measurements at 660 mμ in the Lumetron colorimeter to dry weight.

Incorporation into whole cells. To determine the incorporation of [^{14}C]glycine into whole cells rather than into the trichloroacetic acid precipitate, the following procedure was used. After incorporation, the 3·9 ml. sample was mixed with 1 ml. of cold acetate buffer (0·5 M, pH 4·5) and placed in an ice bath for several minutes. The labelled cells were washed three times with 5 ml. of water, then made up in aq. acetone, added to planchets, dried and counted as usual. The method was based on the assumption that *B. megaterium*, being a Gram-positive organism, would retain its pool of amino acids during the washing procedure.

Oxygen uptake. Conventional manometric methods were used to determine oxygen uptake. The temperature was 37° and air was used as the gas phase. The final volume of liquid was 3·0 ml. and in addition 0·2 ml. of 20% (w/v) KOH was added to the centre well to absorb carbon dioxide.

Materials. Nucleic acids from both *B. megaterium* and *S. aureus*, a gift from Dr E. F. Gale, were prepared by the method of Jones (1953). Dr Gale also supplied incorporation factor prepared from *B. megaterium* nucleic acid by the method of Gale & Folkes (1958). The incorporation factor was assayed by Dr Gale with disrupted staphylococci. It is thought that 1 unit, which is defined as that amount of material which restores the rate of glycine incorporation to that obtained with a saturating dose of nucleic acid, weighs between 1 and 4 μg. in the batch of incorporation factor used here.

The known compounds were tested at a concentration of 100 μg./tube (total volume 3·9 ml.) and their activities were compared with that of 3 mg. of nucleic acid or 2 units of incorporation factor.

RESULTS

Influence of starvation on response to nucleic acid. Incubation of washed *B. megaterium* cells with [^{14}C]glycine in buffered salts mixture led to incorporation of the radioactivity into the cold-trichloroacetic acid precipitate. When nucleic acid from the same organism was added to the reaction mixture, the incorporation was increased. A preliminary starvation period markedly increased the difference in incorporation between the basal and nucleic acid-supplemented tubes, as shown in Fig. 1. Not only did the basal incorporation decrease after starvation but the incorporation with nucleic acid increased. Although 2 hr. of starvation gave an even greater difference, 1 hr. was deemed satisfactory for a workable assay system and was chosen for further work.

Activity of nucleic acid and incorporation factor. Starved cells responded to nucleic acid from both *B. megaterium* and *S. aureus* (Fig. 2), but the former was more potent and stimulated a higher degree of incorporation. This has also been observed by E. F. Gale (personal communication) with the disrupted staphylococcal system. It was generally found that 3 mg. of *B. megaterium* nucleic acid saturated the system, and this concentration was used as a positive control in the remaining experiments. The disrupted staphylococcal system requires about 1 mg. for saturation (Gale & Folkes, 1958).

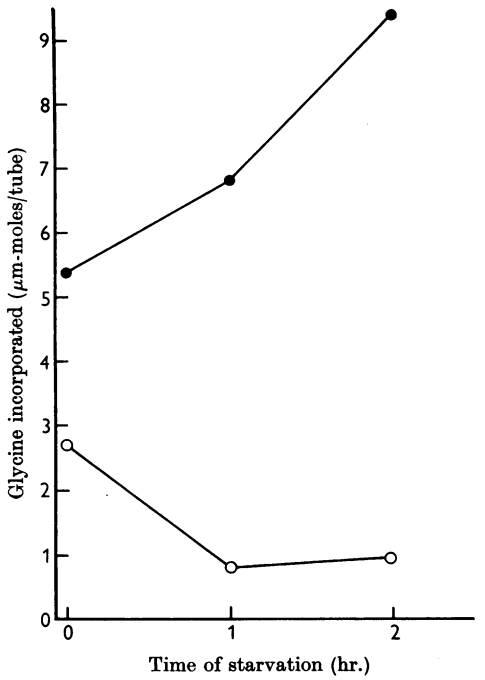

Fig. 1. Effect of starvation on subsequent ability of cells to incorporate glycine in the absence (○) and in the presence (●) of 3 mg. of *B. megaterium* nucleic acid. Cell density remained constant during starvation. Cell density during incorporation was 0·95 mg./tube for all cells. Duration of incorporation was 120 min. A total volume of 3·9 ml. was used.

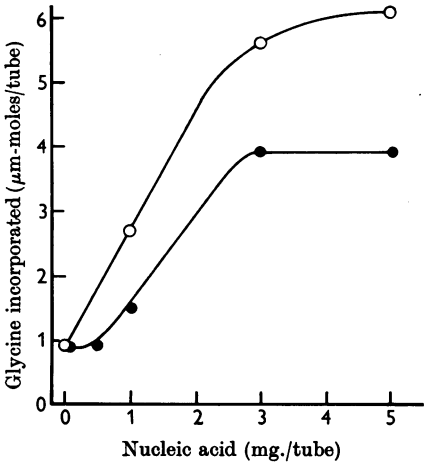

Fig. 2. Stimulation of glycine incorporation by *B. megaterium* nucleic acid (○) and *S. aureus* nucleic acid (●). Cell density was 0·40 mg./tube. Duration of incorporation was 90 min. A total volume of 3·9 ml. was used.

That incorporation factor can replace *B. megaterium* nucleic acid is shown in Table 1. Although 1 unit of incorporation factor replaced 3 mg. of nucleic acid in this experiment, occasionally several units were required. The results correlate well with those of the disrupted staphylococcal assay (Gale & Folkes, 1958).

Commercial nucleic acid samples failed to show significant activity in the assay. This again is in agreement with E. F. Gale's system (personal communication). The materials tested were yeast nucleic acid [Schwartz BioResearch Inc., Mount Vernon, N.Y., U.S.A.; Eastman Organic Chemicals Department (Practical grade); California Foundation, Los Angeles, Calif., U.S.A. (Commercial grade)] and sperm DNA (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.).

Aeration requirements. Aeration requirements were studied by incubating tubes containing 3 mg. of *B. megaterium* nucleic acid under the following conditions: (*a*) static incubation with rubber plugs; (*b*) shaken incubation with rubber plugs; (*c*) shaken incubation with cotton plugs; (*d*) static incubation with rubber plugs but with air being replaced by nitrogen.

The first three conditions gave similar degrees of incorporation, but with nitrogen there was more than 90% inhibition. This suggests that a small amount of oxygen is required for incorporation and that this requirement is satisfied by merely incubating the stoppered tubes in air without shaking. That oxidative phosphorylation is involved in energy generation was shown by the complete inhibition of the incorporation process by 2,4-dinitrophenol (mM).

Effect of cell concentration. Incorporation was directly proportional to cell concentration up to 1·2 mg. dry wt. of bacteria/tube of 3·9 ml.

Effect of ultrasonic vibration. The effect of cellular disruption was tested by treating starved cells for 10 min. in a Raytheon 10 KC sonic oscillator. The ability of the cells to incorporate glycine was completely destroyed by ultrasonic treatment. This is in marked contrast with *S. aureus*, which still retains incorporating ability after ultrasonic treatment (Gale & Folkes, 1958).

Table 1. *Replacement of* Bacillus megaterium *nucleic acid by incorporation factor*

The cell density was 0·65 mg./3·9 ml. Incorporation proceeded for 90 min.

Addition	Glycine incorporation (μm-moles/tube)
None	0·66
Nucleic acid (3 mg.)	4·5
Incorporation factor (0·1 unit)	0·85
Incorporation factor (1 unit)	4·7

Nature of [^{14}C]*glycine binding.* Since glycine radioactivity was isolated in a cold-trichloroacetic acid precipitate, it was possible that we were measuring incorporation into nucleic acid or into the cell wall rather than into protein. To determine how much glycine was entering the nucleic acid fraction, the first cold-trichloroacetic acid precipitate was incubated in hot 5% trichloroacetic acid (85–95°) for 15 min. in place of the usual second cold-trichloroacetic acid wash. After cooling, 5 mg. of carrier RNA was added to the heated tubes and the remainder of the usual washing procedure was carried out. Only 15% of the incorporated radioactivity was removed by hot trichloroacetic acid. It was thus concluded that the major part of the incorporated radioactivity was not in the nucleic acid fraction. McQuillen (1955) noted a similar distribution of incorporated glycine in whole cells of *B. megaterium*, as did Gale & Folkes (1957) with disrupted *S. aureus*.

Likewise, cell-wall biosynthesis was not involved in the incorporation process. Chloramphenicol, which inhibits protein synthesis but not wall formation in *S. aureus* (Hancock & Park, 1958; Mandelstam & Rogers, 1958), markedly inhibited incorporation (Table 2). These results confirm those of Brookes, Crathorn & Hunter (1959), who showed almost complete suppression of glycine incorporation into whole-cell protein of *B. megaterium* by 100 μg. of chloramphenicol/ml. Further proof that wall formation was not involved was obtained by studying the effect of *B. megaterium* nucleic acid and incorporation factor on the incorporation of uniformly labelled L-[^{14}C]leucine, an amino acid not present in the wall of *B. megaterium* (Salton, 1956). Both materials stimulated the incorporation of leucine, as they did in the disrupted staphylococcal system of Gale & Folkes (1958). Both the chloramphenicol and the leucine results make it apparent that the incorporation process studied under the present conditions does not involve wall synthesis but instead incorporation into protein. Intact cells of *S. aureus*, on the other hand, incorporated over 90% of their bound glycine into cell-wall material although, in disrupted cells, the figure dropped to 40% (Gale, Shepherd & Folkes, 1958).

Table 2. *Inhibition of glycine incorporation by chloramphenicol*

The cell density was 1·4 mg./3·9 ml. Incorporation proceeded for 120 min. Figures for glycine incorporation were corrected for contaminating [^{14}C]glycine. This value (0·15 μm-moles) was obtained with a control tube containing trichloroacetic acid during incubation.

B. megaterium nucleic acid (mg./tube)	Chloramphenicol (μg./tube)	Glycine incorporation (μm-moles/tube)	Inhibition (%)
0	0	0·95	—
0	3	0·49	48
0	30	0·24	75
0	300	0·12	87
3	0	5·7	—
3	3	4·0	30
3	30	0·85	85
3	300	0·23	96

Intracellular accumulation of [^{14}C]*glycine.* To determine whether a pool of [^{14}C]glycine accumulates intracellularly, the radioactivity of whole cells was compared with that of the trichloroacetic acid precipitate at various times during the incubation period. At no time was the whole-cell radioactivity significantly greater than the precipitable activity, indicating that the labelled glycine is rapidly incorporated into protein once it gets into the cell.

Inhibition by 4,5,6-trichlorobenzimidazole. To further determine the degree of similarity between the intact *B. megaterium* assay and the disrupted *S. aureus* system, the effect of 4,5,6-trichlorobenzimidazole was studied. This compound was found by Gale & Folkes (1957) to be an effective inhibitor of glycine incorporation by disrupted staphylococci, showing 50% inhibition at a concentration of 0·4 mM. In the *B. megaterium* system, 4,5,6-trichlorobenzimidazole (0·34 mM) completely inhibited incorporation.

Activity of ribonucleosides. A final test of the similarity between the two assays involved the testing of ribonucleosides and their derivatives. Gale (1959) reported that many ribonucleosides (but not ribonucleotides, their free bases or ribose) were active in the disrupted staphylococcal assay at concentrations of 0·1–1·0 mM, although it is clear from other considerations that they are not the active components of the incorporation factor. We have obtained similar activity with *B. megaterium*. We have found uridine, inosine, purine riboside and kinetin riboside to be active, whereas uridylic acid, uracil and ribose are inactive. Further, our system is similar to the staphylococcal assay in that there is a curious variability from experiment to experiment with ribonucleosides [for a discussion of this effect, see Gale (1959, p. 93)].

Activities of sugars and sugar alcohols. Since no exogenous carbon or energy source is included in the standard *B. megaterium* assay, whereas the disrupted staphylococcal system (Gale & Folkes, 1958) includes hexose diphosphate and ATP, we first studied the ability of these two phosphorylated materials to replace incorporation factor. It was not surprising that these compounds gave no stimulation as the test system employed intact cells. In fact, [^{14}C]glycine incorporation was somewhat inhibited when these two compounds were used at the concentration recommended for dis-

rupted *S. aureus*. However, when non-phosphorylated hexoses were tested, it was found that glucose and fructose could replace incorporation factor but that galactose, mannose and 2-deoxyglucose had no activity. The disaccharides, lactose and maltose, were also inactive; sucrose, however, showed partial activity. Starch had no activity. Pentoses (arabinose, xylose, ribose, deoxyribose, rhamnose) were also inactive, as were the sugar alcohols, erythritol, mannitol and sorbitol. Glycerol, on a weight basis, was more active than glucose (Table 3). However, on a molar basis glycerol and glucose were equivalent. No other compound was found to be more active than glycerol. Compounds closely related to glycerol, e.g. glyceraldehyde and α-glycerophosphate, were inactive.

A large number of intermediates of carbohydrate metabolism and related compounds were tested and found to be inactive. This list included phosphorylated sugars, 6-*O*-acetylglucose, pyruvate, succinate, citrate, acetate, aconitate, α-oxoglutarate, malate, fumarate, ethyl oxaloacetate, glyoxylate, 2-oxogluconate, 5-oxogluconate, glucuronate, galacturonate, galactosamine, glucosamine, *N*-acetyl-D-glucosamine, mesaconate, acetoin, hexanoate, butyrate, glycollate, dipicolinate, pimelate, formate, glutarate, isovalerate, isobutyrate, dihydroxyacetone and ethanol. The only compound possessing activity in this category was D-glucono-δ-lactone, which, however, was less potent than either glucose or glycerol.

Since lysed cells of *B. megaterium* oxidize several of the above-mentioned 'inactive' intermediates (Storck & Wachsman, 1957), we tested whether their inactivity in the incorporation assay was due to their inability to penetrate the washed and starved intact cell rapidly. Only those compounds which led to oxygen uptake, demonstrated manometrically, were capable of stimulating incorporation of glycine. This suggests that the inactivity of many of the compounds may be due to their inability to penetrate the cell rapidly.

It was of interest to determine whether cells grown with an 'inactive' carbon source could be induced to use such a compound for glycine incorporation. Cells were grown simultaneously in the basal growth medium and in the same medium with mannitol replacing glucose. After harvesting, the two types of cells were washed, starved and tested for oxygen uptake and for stimulation of incorporation by glucose and mannitol. Table 4 shows that cells grown on mannitol are capable of oxidizing mannitol rapidly and utilizing it for incorporation of glycine. Glucose is active for mannitol-grown cells as well as for glucose-grown cells.

The activity of glycerol and glucose suggested that the stimulation by nucleic acid preparations might be due to contamination with such small molecules. Indeed, overnight dialysis of *B. megaterium* nucleic acid against distilled water in prewashed dialysis tubing led to passage of the active material through the bag. Paper chromatography of the diffusible material in propan-2-ol–ammonia (7:3, v/v) indicated that the active material was glycerol. However, we failed to extract the activity from nucleic acid into absolute ethanol. Thus it appears that the potency of nucleic acid was due to bound glycerol.

Activity of amino acids. Addition of an amino acid mixture lacking glycine to the *B. megaterium* system gave an apparent inhibition of glycine incorporation. However, when the amino acids were tested individually, the following five were found to have stimulatory activity: DL- or L-glutamic acid, L-proline, L-glutamine, L-arginine hydrochloride and DL-aspartic acid. The degree to which they could replace incorporation factor, however, was poorly reproducible from experiment to experiment—a situation reminiscent of the activity of ribonucleosides. These amino acids never completely replaced incorporation factor or media even at the high concentration of 1 mg./tube. However, in many experiments they exhibited half-maximal incorporation at a concentration of 100 μg./tube.

Activity of other compounds. No other compounds studied showed any activity. Included among these inactive compounds were the B vitamins,

Table 3. *Comparison of glycerol and glucose*

The cell density was 1·3 mg./3·9 ml. Incorporation proceeded for 90 min.

Glycerol (μg./tube)	Glucose (μg./tube)	Glycine incorporation (μm-moles/tube)
0	0	0·49
1·5	0	0·93
7·5	0	3·5
15	0	4·1
0	1·5	0·68
0	7·5	1·6
0	15	3·0

Table 4. *Effect of growth substrate on incorporation*

The cell density of the glucose-grown cells was 0·65 mg./3·9 ml. That of the mannitol-grown cells was 0·68 mg./3·9 ml. The incorporation proceeded for 90 min.

Type of cells	Addition	Glycine incorporation (μm-moles/tube)
Glucose-grown	None	0·20
	Glucose (100 μg.)	3·4
	Mannitol (100 μg.)	0·18
Mannitol-grown	None	0·23
	Glucose (100 μg.)	1·6
	Mannitol (100 μg.)	1·4

ferrichrome, DL-carnitine hydrochloride, biocytin, choline chloride, ascorbic acid, reduced glutathione, inositol monophosphate, betaine hydrochloride, creatinine, creatine, cytochrome *c*, putrescine dihydrochloride, sarcosine, EDTA (disodium salt), haematoporphyrin, orotic acid, ethanolamine, DL-plus allo-cystathionine, hippuric acid, L-ergothioneine hydrochloride, haemoglobin, 4-aminoimidazole-5-carboxamide hydrochloride, TPN and DPN.

DISCUSSION

The *B. megaterium* assay developed by us for the estimation of incorporation factor appears to correlate well with the disrupted staphylococcal system of Gale & Folkes (1958). Both systems respond to bacterial nucleic acids but not to commercial yeast nucleic acids, are inhibited by 4,5,6-trichlorobenzimidazole and give similar responses to ribonucleosides. The activity in the bacterial nucleic acid appears to be due to bound glycerol, which can be removed by dialysis but not by extraction with ethanol. We have tested a large number of compounds, of which only glycerol, glucose and fructose proved active. They fully replaced incorporation factor when added to the reaction vessels at a concentration of 100 μg./tube (25·6 μg./ml.). Warburg studies indicated, however, that many of the inactive compounds were not metabolized during the short incubation period used in the incorporation assay, possibly because they failed to enter the intact cells. The results for the inducibility of incorporating activity with mannitol suggest that some of these compounds might also have been active if the cells had been 'trained' to utilize them. Whether or not this narrow specificity of activity is more apparent than real, one effect of practical importance has been the identification of a 5-month-old sample of incorporation factor as glycerol by Kuehl *et al.* (1960).

We have no evidence against the hypothesis that, in our assay, glycerol is merely acting as an energy source. However, the studies of Gale & Folkes (1962) with intact *Escherichia coli* cells speak against such an interpretation. Furthermore, E. F. Gale (personal communication) has found that, whereas fresh incorporation factor displays a linear type of glycine incorporation curve using the disrupted *S. aureus* system, aging converts it into one displaying a lag phase typical of glycerol.

SUMMARY

1. The development and details of a simple and reproducible assay for the 'incorporation factor' are described. It is based on the stimulation of [^{14}C]glycine incorporation into intact *Bacillus megaterium* cells by the incorporation factor described by Gale & Folkes (1958).

2. The characteristics of the assay show marked similarities to those of the more difficult and laborious disrupted *Staphylococcus aureus* system in the following respects: (*a*) activity of nucleic acids from *B. megaterium* and *S. aureus* and inactivity of commercial nucleic acid preparations; (*b*) inhibition by 4,5,6-trichlorobenzimidazole; (*c*) activity of ribonucleosides and inactivity of ribose, free bases and ribonucleotides. A further improvement over the disrupted staphylococcal system lies in the observation that no significant portion of the glycine incorporated by the intact cells of *B. megaterium* enters the cell-wall fraction.

3. Of a large number of known compounds tested, only glycerol, glucose and fructose were able to replace the incorporation factor completely. On a weight basis the most active compound was glycerol. Permeability difficulties may account for the inactivity of many of the compounds.

4. The activity of nucleic acid prepared from *B. megaterium* appears to be due to bound glycerol, which can be removed by dialysis but not by extraction with ethanol.

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