

THE BINDING OF FATTY ACIDS BY SERUM ALBUMIN,  
A PROTECTIVE GROWTH FACTOR IN  
BACTERIOLOGICAL MEDIA

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It has been reported from this laboratory that while polyoxyethylene sorbitan monooleate (marketed under the trade mark Tween 80<sup>1</sup>) facilitates submerged growth of tubercle bacilli in liquid media, initiation of growth by small inocula requires addition of serum albumin (1, 2). Other workers, using media without Tween, had previously noted that this protein promotes growth of tubercle bacilli (3, 4); in addition, whole serum has been employed for many years in a variety of media designed for the cultivation of tubercle bacilli and other bacteria.

At first we assumed that albumin, like most known growth factors, contributed a nutritive which was absorbed by the tubercle bacilli. After studying the mechanism of action of albumin, however, it was reported briefly (5) that some improvement in growth of small inocula was provided by the addition to the medium of albumin within a cellophane bag, even though this prevented contact between the bacteria and the protein; addition to the medium of the dialysate of albumin, however, was without effect. These observations were taken to indicate the presence in the medium of a dialyzable inhibitor which was bound to albumin. Subsequent work, published in a preliminary note (6), showed that the chief inhibitor against which albumin protects the tubercle bacilli is unesterified oleic acid present in the commercial product Tween 80, and further released from it during incubation.

Since oleic acid was not found to dialyze freely through cellophane, the interpretation of the earlier dialysis experiments is open to some doubt. Nevertheless, the conclusion, drawn from them, that albumin acts protectively by binding, not only is confirmed but is much more strikingly demonstrated by experiments involving albumin and oleic acid. These latter experiments will be described in the present paper, along with evidence that Tween 80, when freed of unesterified oleic acid, is not only non-toxic but even protective.

EXPERIMENTAL

*Methods*

Bacteriological experiments were performed as previously described (2), using 5 ml. of medium in a metal-capped wide test tube (25 × 250 mm.). The medium consisted of a

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<sup>1</sup> Furnished through the courtesy of the Atlas Powder Company, Wilmington.

mineral mixture buffered by phosphate at pH 7.0, enzymatic casein hydrolysate, Vegex (yeast autolysate), and Tween 80 (0.05 per cent unless otherwise specified). After autoclaving at 120° for 10 minutes and addition of sterile glucose, 5 per cent neutralized bovine serum albumin (fraction V) (7), sterilized by filtration, was added aseptically to yield a final concentration of 0.1 per cent (unless otherwise specified). The other protein solutions tested were also sterilized by passage through sintered glass filters (Corning UF) and added aseptically. After inoculation the tubes were incubated at 37°C. The inoculum was a standard laboratory strain of a human tubercle bacillus, H37Rv, which had been grown for many passages in this Tween-albumin medium. The size of the inocula designated in the tables represents volumes of a 7 to 10 day old culture containing approximately 1 mg. of moist organisms (0.2 mg. dry weight) per ml. Growth is recorded in the tables in terms of a visual estimate ranging from 0 (no visible growth) to + + + + (full growth, approximately 2 mg. moist weight per ml.).

The oleic acid used was a commercial preparation (Eimer and Amend). The bovine serum albumins (fraction V and crystalline albumin) were obtained from the Armour Laboratories.<sup>2</sup>

#### *Promotion of Growth by Undenatured Albumin*

The favorable effect of serum albumin on the growth of small inocula of the tubercle bacillus was not produced by comparable concentrations of a variety of other proteins: serum globulins (fractions II, III-1, and IV-1), gelatin, protamine, casein, ovalbumin, gliadin, or edestin. A very slight effect was exerted by  $\beta$ -lactoglobulin. Growth was promoted by serum albumin following dialysis, but not by its dialysate or by serum albumin following trypsin hydrolysis (sufficient to remove approximately 90 per cent of its precipitability by trichloroacetic acid); in this connection appropriate control experiments were carried out to determine that the loss of growth promotion following trypsin hydrolysis was not caused by any bacteriostatic effect of the trypsin or the hydrolysate. Finally, the property of facilitating growth was shown to be destroyed following heating of 5 per cent albumin to 100°C., whether the albumin was coagulated or was heated under conditions which avoided coagulation (neutralized, no salt added). Heating to 56°C. for 30 minutes, however, did not destroy the property. Some of these experiments are presented in Table I.

It was concluded that the binding property depends upon the native, undenatured configuration of the albumin molecule. Since it seemed unlikely that a bacterium could derive specific nutritive benefit from a whole protein molecule, the thought arose that the albumin, which is known to bind a variety

<sup>2</sup> Crystalline hen ovalbumin was furnished through the kindness of Dr. Gertrude Perlmann, and crystalline  $\beta$ -lactoglobulin (prepared by Dr. G. Haugaard) through the kindness of Dr. William Stein, of The Rockefeller Institute for Medical Research. The products of human plasma fractionation employed in this work were furnished through the kindness of the Department of Physical Chemistry, Harvard Medical School, and were developed under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

of small molecules (8-10), might be functioning as a protective rather than a nutritive growth factor.

*Protection by Albumin against Oleic Acid*

When it was found that 3 per cent of the oleic acid in commercial Tween 80 (0.6 per cent of the Tween by weight) is unesterified (6, 11) and that the tubercle bacillus is sensitive to extremely low concentrations of free oleic acid (12)

TABLE I  
*Effect of Various Proteins on Growth of Tubercle Bacilli in a Medium  
Containing 0.05 Per Cent Unpurified Tween 80*

Substance added	Concentration	Inoculum (moist weight)	
		10 <sup>-3</sup> mg.	10 <sup>-4</sup> mg.
		Growth at 15 days	
	<i>per cent</i>		
Control.....		0	0
Bovine crystalline albumin.....	0.2	++++	++
Bovine albumin (fraction V).....	0.2	++++	++
Trypsin-digested bovine albumin.....	0.2	0	0
Trypsin-digested bovine albumin.....	0.5	0	0
Trypsin-digested bovine albumin + undigested bovine albumin.....	0.2		
	+0.2	++++	++
Dialyzed bovine albumin.....	0.2	++++	++
Human albumin (fraction V).....	0.2	+++±	+±
Human globulin (fraction II).....	0.2	0	0
Human globulin (fraction II).....	0.5	0	0
Ovalbumin.....	0.2	0	0
Ovalbumin.....	0.5	0	0
Bovine albumin heated to 56°.....	0.2	++++	++
Bovine albumin heated to 100°.....	0.2	0	0

(small inocula being sensitive to less than 1  $\mu$ g. per ml.—*cf.* Table IV), experiments were undertaken to test whether oleic acid might be the substance against which albumin protects the tubercle bacillus. Table II shows that albumin in a wide range of concentration will protect very small inocula of tubercle bacilli against oleic acid up to 1 per cent of the weight of albumin, and moderate inocula against 2 per cent. From these results it can be calculated that the concentration of albumin present in the usual medium (0.1 per cent) is easily capable of protecting the organism against the concentration of oleic acid (3  $\mu$ g. per ml.) which is introduced by the addition to the medium of 0.05 per cent Tween 80.

Albumin similarly protected tubercle bacilli against the toxic effect of added stearic acid.

TABLE II  
*Protection by Serum Albumin against the Bacteriostatic Effect of Oleic Acid on Tubercle Bacilli*

Oleic acid	Albumin	Inoculum (moist weight)		
		10 <sup>-7</sup> mg.	10 <sup>-4</sup> mg.	10 <sup>-4</sup> mg.
<i>per cent</i>	<i>per cent</i>	Growth at 14 days		
Medium containing 0.05 per cent Tween 80				
0	0.1	++++	++++	++
0.001	0	++++	0	0
	0.05	++++	++±	0
	0.1	++++	++++	0
0.002	0.05	++++	0	0
	0.1	++++	++	0
	0.2	++++	++++	+
0.004	0.1	++++	0	0
	0.2	++++	+++	0
	0.4	++++	++++	++
0.008	0.2	0	0	0
	0.4	++++	+++	0
	0.8	++++	++++	±±
	1.6	++++	++++	±±
0.016	0.4	—	0	0
	0.8	++++	+++	—
	1.6	++++	++++	0
Medium without Tween 80				
0.004	0.1	0	0	0
	0.2	++++	+++	+
	0.4	++++	+++	+

Growth in the medium without Tween was flocculent, so the figures are not comparable to those obtained in the medium with Tween.

*Elimination of Bacteriostatic Effect of Tween 80 by Purification*

Although the bacteriostatic property of the commercial product Tween 80 could thus be accounted for by its content of free oleic acid, direct proof that the Tween 80 molecule itself is not bacteriostatic required the preparation of material essentially free from fatty acid. The method of purification is de-

scribed in another paper (11); it yields a product containing less than 0.1 per cent unesterified oleic acid by weight. Table III shows that this purified Tween 80 is non-toxic for tubercle bacilli in high concentrations. From the data of Table IV, in the next section, it may be seen that inocula as small as  $10^{-7}$  mg. will grow in the presence of 0.05 per cent purified Tween 80 without addition of albumin.

The purification of Tween 80 has been indispensable for elucidating the mechanism of action of albumin, but it is not anticipated that the use of purified Tween 80 will replace the inclusion of albumin in the medium for most

TABLE III  
*Elimination of Bacteriostatic Effect of Tween 80 by Purification*

Tween 80	Inoculum (moist weight)		
	$10^{-2}$ mg.	$10^{-3}$ mg.	$10^{-4}$ mg.
	Growth at 9 days		
<i>per cent</i>			
	Unpurified Tween, no albumin		
0.05	++±	+±	±
0.1	+++	+	0
0.2	+++	0	0
0.4	+++	0	0
0.8	+++	0	0
	Fatty acid-free Tween, no albumin		
0.05	++±	+±	—
0.1	+++	++	+±
0.2	+++	++	+±
0.4	+++	++	+±
0.8	+++±	++	+±
	Unpurified Tween, 0.1 per cent albumin		
0.05	+++±	+±±	+±

purposes. Not only is it difficult to avoid traces of fatty acids, but the spontaneous hydrolysis of Tween 80 (11) is so fast that minimal inocula ( $10^{-8}$  mg. moist weight, 2 to 3 cells) cannot develop in the absence of albumin, even with freshly purified Tween 80.

While the bacteriostatic action of Tween 80 on the tubercle bacillus is entirely explained by its content of unesterified oleic acid, there are unknown bacteriostatic factors in some of the other Tweens. Several lots of Tween 60, for example, the analogous ester of stearic acid, yielded barely detectable amounts of free fatty acid (approximately  $\frac{1}{6}$  as much as Tween 80), yet these lots varied widely in their inhibitory effect on small inocula of the tubercle bacillus. It was determined that added stearic acid was quantitatively recovered from Tween 60 solution by the analytical method, and that stearic acid is less toxic

to the tubercle bacillus than oleic acid. The toxic effect of certain lots of Tween 60, then, is not due to their content of free fatty acid. It may be noted here that hydrogen peroxide is used in the manufacture of the Tweens.

TABLE IV  
*Protection by Tween 80 against the Bacteriostatic Effect of Oleic Acid on Tubercle Bacilli*

Fatty acid-free Tween 80	Oleic acid	Inoculum (moist weight)					
		10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup> mg.
		Growth at 15 days					
<i>per cent</i>	<i>µg./ml.</i>						
		No albumin					
0	0	++	+	+	±	0	0
	1	0	0	0	0	0	0
	3	0	0	0	0	0	0
	10	0	0	0	0	0	0
0.01	0	—	+++	++	++	+±	+
	1	—	+++	++	+±	+	±
	3	+++±	±	0	0	0	0
	10	—	0	0	0	0	0
0.05	0	—	+++±	+++	++	++	++
	1	—	+++±	+++	++	+±	+
	3	+++±	+++±	+++	+±	+±	±
	10	0	0	0	0	0	0
0.2	0	++++	++++	+++±	—	++	++
	1	++++	++++	+++±	+±	++	+
	3	—	++++	+++±	++	+±	+
	10	++++	±	0	0	0	0
		0.1 per cent albumin					
0.05	0	++++	++++	++++	+++	+±	++

*Protection by Tween 80 against Oleic Acid*

The "fatty acid-free" Tween 80 actually retains approximately  $\frac{1}{10}$  the free fatty acid originally present; *i.e.*, 0.06 per cent by weight of oleic acid (6, 11). Nevertheless, when the toxic concentration of this purified material was sought, it was found that even 0.8 per cent purified Tween 80 showed no inhibitory effect on growth (Table III), in contrast to the marked effect of the smaller amount of free fatty acid in 0.05 per cent unpurified Tween 80. This phenomenon was explained by the observation that Tween 80 itself offers a certain amount of protection against the inhibitory effect of free oleic acid (Table IV). The Tween 80 presumably combines reversibly with free fatty acid, as does serum albumin, but is less effective per unit concentration than the albumin.

Because of this protective effect, Tween 80 need not be absolutely free of unesterified acid in order to be effectively fatty acid-free for bacteriological work; it need only have a proportion of fatty acid to ester which is below a certain critical level. Baker, Harrison, and Miller (13) have likewise shown that an unionized surface active agent rather similar to the Tweens (Demal, a mixture of polyglycerol esters), and natural phospholipids as well, prevent inhibition of bacterial metabolism and sterilization by either cationic or anionic synthetic detergents.

That there is some sort of interaction between Tween and fatty acids could be predicted from the capacity of Tween to disperse large amounts of fatty acids and various other lipids in clear solution in water.

#### *Effect of Albumin on Surface Growth*

When a tube of Long's synthetic medium (which contains no Tween) was inoculated for surface growth by transfer of a small piece of pellicle of the tubercle bacillus H37Rv grown for 4 weeks on the same medium, growth at the edge of the inoculum was first visible in 4 to 6 days. When 0.1 per cent serum albumin was added to this medium, pellicle growth was initiated much more rapidly, being visible within 2 to 3 days of inoculation. It appears likely that the delay and occasional failure in the initiation of surface growth on simply synthetic media may be due to contamination by traces of fatty acid. The delay may be accounted for by the observation that tubercle bacilli can absorb and presumably metabolize fatty acids in bacteriostatic concentrations, and then multiply after elimination of this excess of fatty acid (13 a).

The addition of oleic acid as well as albumin further slightly stimulated surface growth. This observation parallels the stimulating effect of long chain fatty acids, in the presence of albumin, on other modes of growth of tubercle bacilli, submerged in a liquid medium or on the surface of a solid medium (12).

It appears unlikely that the addition of albumin would be of practical value in the preparation of tuberculin, for which surface cultures of the tubercle bacillus are largely used, since the inclusion of a foreign protein would be undesirable and the use of very large inocula permits quite regular initiation of growth.

#### *Binding Capacity of Serum Albumin for Oleic Acid*

(a) *Bacteriological*.—With fatty acid-free Tween 80 it was possible to study quantitatively the binding capacity of albumin for oleic acid in the medium. Table II showed that serum albumin is able, over a wide concentration range (0.1 to 1.6 per cent), to bind 1 to 2 per cent of its weight of oleic acid firmly enough to neutralize the bacteriostatic effect of the fatty acid. This corresponds to 3 to 6 molecules of oleic acid (molecular weight 282) per molecule of albumin (molecular weight 70,000).

While these bacteriological studies clearly suggest that serum albumin can bind oleic acid in the proportion indicated, an element of uncertainty arises from the possibility that (a) the ratio obtained may represent a competition for the surface of the bacteria, rather than a physicochemical equilibrium in homogeneous solution; and (b) albumin, oleic acid, or impurities in either may have unknown effects on the metabolism of the tubercle bacillus which influence the result. The interaction between albumin and oleic acid was therefore studied by two further methods: inhibition of the hemolytic effect of oleic acid on red blood cells, and inhibition of the opalescence of emulsions of oleic acid.

(b) *Hemolytic*.—Table V shows that serum albumin protects red cells from hemolysis by oleic acid, and that the amount of fatty acid bound by the albumin is between 2 and 4 per cent, somewhat larger than that observed in the bacteriological experiments described above. Crystalline  $\beta$ -lactoglobulin protected the red cells from  $\frac{1}{2}$  to  $\frac{1}{4}$  as much oleic acid as did serum albumin; ovalbumin, protamine, and gelatin offered no protection.

Tween 80 proved to be approximately as effective as albumin in protecting red cells from hemolysis during incubation with added oleic acid for 30 minutes at 37°C.; however, Tween 80 itself was very hemolytic when the incubation was continued overnight in the refrigerator.

No further effort was made to refine the hemolytic method, which may be capable of much greater precision than is indicated by Table V. It was observed that sheep red cells demand a rather large amount of oleic acid (approximately 1 per cent of the weight of cells) to produce hemolysis. The sensitivity of the method as a test for unbound oleic acid is consequently greater the lower the concentration of red cells.

(c) *Chemical*.—It was not found possible to attain dialysis equilibrium with oleic acid across a cellophane membrane; the binding of oleic acid by albumin could therefore not be measured by this method, as had been done with various other compounds bound by albumin (8, 14, 15). Another thermodynamically sound method of measuring the interaction, however, is the increase in solubility of oleic acid in the presence of albumin. Since exceedingly small amounts of sodium oleate become opalescent when added to a neutral phosphate buffer, the binding capacity of serum albumin may be simply estimated by measuring the ratio of oleic acid to albumin which produces opalescence at various albumin concentrations.

This principle is illustrated in Table V, which indicates not only the effect of albumin on hemolysis, but the clarity or opalescence of the mixtures of oleate and albumin before addition of red cells. It is seen that the binding capacity of albumin is between 2 and 4 per cent, the variation depending on the roughness of serological dilutions. Further experiments indicated a binding capacity of approximately 3 per cent, as judged by the extinction of opalescence. This value corresponds to a ratio of approximately 9 molecules of oleic acid (molecular



weight 282) per molecule of serum albumin (molecular weight 70,000). It may be noted that the binding capacity of albumin observed in the bacteriological tests was only 3 to 6 molecules of oleic acid. The discrepancy is not surprising, since (1) the bound oleic acid is undoubtedly in equilibrium with free acid, the concentration of free acid increasing with increasing saturation of the protein, and (2) the bacteria are apparently sensitive to a lower concentration of free acid than that found in an aqueous solution in equilibrium with an excess of solute (as indicated by opalescence).

TABLE V

*Protection by Serum Albumin against Hemolysis by Oleic Acid*

Fresh sheep red blood cells were washed 4 times in neutral phosphate-saline buffer (0.14 M NaCl, 0.02 M phosphate, pH 7.0), and suspended in this buffer in a concentration of 0.4 per cent by volume. A 1 per cent solution of oleic acid in dilute NaOH was neutralized with concentrated HCl to incipient turbidity. Bovine serum albumin (fraction V) was neutralized in 5 per cent solution. Dilutions of the albumin and the oleate solutions were prepared in the phosphate-saline buffer. Volumes of 0.5 ml. of the albumin dilutions were added to 1.0 ml. of the oleate dilutions in small test tubes; 0.5 ml. of the red cell suspension was added, and the tubes were incubated in a 37°C. water bath for 30 minutes. In this experiment the results indicated were unchanged after further incubation overnight in the refrigerator, although in some other experiments certain borderline tubes developed hemolysis overnight. The concentrations noted are the final concentrations in the mixtures. Also indicated in the table is the clarity or opalescence of each mixture of albumin and oleic acid before addition of red cells.

Concentration of albumin <i>per cent</i>	Concentration of oleate, <i>per cent</i>				
	0.05	0.02	0.01	0.005	0.002
1.0	0(cl)	0(cl)	0(cl)	0(cl)	0(cl)
0.5	H(op)	H(op)	0(cl)	0(cl)	0(cl)
0.25	—	—	0(cl)	0(cl)	0(cl)
0.1	—	—	H(op)	H(op)	0(cl)
0.05	—	—	H(op)	H(op)	0(cl)

H = hemolysis.

0 = no hemolysis.

op = opalescent mixture of oleate and albumin.

cl = clear mixture of oleate and albumin.

Serum albumin has a greater capacity for tightly binding long chain fatty acids than any other substances tested. Human serum globulin (fraction II), protamine, gelatin, and crystalline ovalbumin, tested in 3 per cent solution, had no effect on the opalescence of oleic acid. This absence of binding explains the earlier observation that these proteins exerted no beneficial effect on the cultivation of tubercle bacilli. Crystalline  $\beta$ -lactoglobulin quenched the opalescence of approximately half as much oleic acid per unit weight of protein as did serum albumin.

## DISCUSSION

It has been established that the predominant if not the only rôle of serum albumin in permitting initiation of growth of tubercle bacilli by small inocula is to function as a protective rather than a nutritive growth factor.<sup>8</sup> In the media under present consideration the growth inhibitor bound by the albumin is free oleic acid, largely contributed by the Tween 80. Similarly, Gould, Kane, and Mueller (17) have recently reported that starch promotes growth of gonococci on solid media by binding traces of an inhibitor present in the agar; this inhibitor was found by Ley and Mueller (18) to be oleic acid. It is now possible to interpret in terms of a protective growth factor the older observation of Uyei (19) that the value of potato extract in media for the diagnostic cultivation of tubercle bacilli is due to the soluble starch present in this extract. The affinity of starch for fatty acids (20) is probably less than that of albumin, for we have not found starch nearly as effective as albumin in promoting growth of tubercle bacilli in solid media containing Tween 80.

The binding property of serum albumin undoubtedly contributes to the value of blood, serum, or ascitic fluid in various "enriched" media. The commercial availability of pure serum albumin now permits better controlled utilization of this property. Since fatty acids are ubiquitous contaminants of glassware, cotton plugs, and reagents, and since many bacteria (particularly Gram-positive and acid-fast species) are very sensitive to fatty acids, it appears probable that the property of albumin here described will be useful in obtaining reproducible growth or growth from minimal inocula of organisms other than the tubercle bacillus. In the field of microbiological assay of vitamins and amino acids, for instance, it has been pointed out that irregularities in the growth response of *Lactobacillus casei* are frequently caused by contamination by fatty acids (21, p. 85). Indeed, even outside the domain of bacteriology it has been observed by Clarke (22) that commercial Tween 80 depresses the respiration of red blood cells parasitized by *Plasmodia*; the toxic effect may either be corrected by the addition of serum albumin or avoided by the use of fatty acid-free Tween 80.

The reversible binding of fatty acids by albumin permits the fatty acids to serve as nutrient growth factors which enhance the growth of tubercle bacilli (especially of the avian type) and other bacteria (12), whereas in the absence

<sup>8</sup> The initiation of growth is facilitated equally well by crystalline bovine serum albumin, but the less pure amorphous fraction V also has a further slight enhancing effect on the richness of growth which has been traced to a heat-stable, dialyzable factor (12). In addition to its desirable effects, fraction V has the undesirable property of rendering the medium unstable through the contamination of the albumin by a trace of lipase, which slowly hydrolyzes the Tween and releases free oleic acid. The lipase may be inactivated by heating the albumin at 56°C. for 30 minutes, which does not destroy the desirable binding property of the albumin, or by adding NaF. The albumin then permits regular initiation of growth by smaller inocula (two bacterial cells) than have otherwise been effective (16).

of albumin the fatty acid is bacteriostatic. The albumin apparently "buffers" the fatty acid so that the concentration of free fatty acid is very low, below the bacteriostatic level, while the bound acid constitutes a reserve which replaces the fatty acid withdrawn by the bacteria. Esterification of the fatty acid plays a similar rôle, except that it appears possible that the bacterium absorbs the free fatty acid in the one case, and the ester in the other (16).

In this study serum albumin has served as a useful chemical reagent, now conveniently available as a result of the wartime plasma fractionation program. But the implications of the results presented here are not limited to the field of microbial nutrition, for albumin is a substance of biological origin. The relative uniqueness of this property of albumin must be stressed,  $\beta$ -lactoglobulin being the only other protein found so far to show even a fraction of the same affinity. This uniqueness has also been reported for the weaker interaction of albumin with short chain fatty acids (23), and is probably true of its interactions with many other organic anions, including sulfonamides (14), anionic dyes (15), and a variety of drugs and other compounds (reviewed in 8-10). Since fatty acids are physiologically the most important members of the group of bindable substances studied thus far, considerations of teleology (or, more precisely, of evolutionary survival value) suggest that this property of albumin probably serves a useful physiological function, such as transport of materials in the blood and protection of animal cells against toxic effects of various substances (*e.g.*, hemolysis by free fatty acids). The combination of cytotoxic and nutritive properties of fatty acids, and the modification of the balance of these properties by serum albumin, may find quite a close analogy in the animal organism to the effects observed in bacteriological culture media.

The physiological significance of the binding capacity of serum albumin has been discussed in greater detail elsewhere (10). The affinity of serum albumin for chemotherapeutic agents with anionic groups is of special practical importance. The failure of a large number of bacteriostatic substances (including fatty acids) to be effective chemotherapeutic agents *in vivo* can be attributed partly to their affinity for serum albumin. One cannot overemphasize the importance of including serum or albumin in the medium when testing potential chemotherapeutic agents *in vitro*. The inefficacy of penicillin K *in vivo*, for instance, as compared with penicillins G, F, and X, (all having been standardized *in vitro* in the absence of albumin), has recently been accounted for by the much greater extent to which serum albumin binds penicillin K (24, 24 a). In connection with the present work it is of interest to point out that the penicillins consist essentially of a hydrocarbon chain or ring separated by a pair of unusual heterocyclic rings from a carboxyl group. Penicillin K, which is bound approximately 8 times as extensively as penicillin dihydro F, differs from the latter simply by having two more carbon atoms on its hydrocarbon chain; *i.e.*, resembling a longer chain fatty acid.

Although the inhibitory effect of serum on the antibacterial and hemolytic action of soaps was observed at least as early as 1907 (25-27), and the interaction of proteins with drugs, indicator dyes, etc., has been known to biologists for decades, it is only in very recent years that these reversible reactions have begun to be seriously used as physicochemical tools for studying protein structure (15, 28). Our knowledge of the structures responsible for these reactions is as yet exceedingly scanty. The failure of certain proteins to bind oleic acid cannot be ascribed to the lack of any known components of serum albumin. The special capacity of albumin therefore implies the presence on the surface of the molecule of regions where the specific configuration of the amino acid residues leads to interaction with the fatty acid. This view is supported by the fact that binding demands the native, undenatured configuration of the albumin.

The type of configuration which might be predicted to be suitable for binding a long chain fatty acid would be a quaternary nitrogen atom, binding the oppositely charged carboxyl ion, adjacent to several non-polar residues which would attract the non-polar chain of the fatty acid by van der Waals forces. Since the most distinctive feature of the composition of serum albumin is its unusually high content of lysine and leucine, it is tentatively suggested that albumin may have a number of lysine residues each of which is adjacent to several leucine or other non-polar residues. (For a similar suggestion, *cf.* 29.) The importance of the regions adjacent to the quaternary nitrogen is emphasized by the absence of binding by the basic protein protamine, which contains approximately 90 per cent arginine and hence an abundance of quaternary nitrogens.

The assumption that the quaternary nitrogen of lysine is involved in the binding is strengthened by the observation of Klotz (28) that pH affects the binding of anionic dyes by albumin only in the region (above pH 9) where the  $\epsilon$ -amino nitrogen of lysine loses its charge. Only a fraction of the approximately 60 lysine groups of albumin would be needed to account for the binding of 9 oleic acid molecules. Indeed, until further information is obtained, we cannot be certain that as many as 9 binding sites are present on each albumin molecule, for the tendency of fatty acids to form micelles in aqueous solution may also apply to their interaction with albumin. This seems unlikely, however, in the very dilute solutions under consideration. The binding of shorter chain fatty acids by albumin, although less tight, is also reported to involve at least 9 molecules, in ultrafiltrates from 0.005 M caprylate (30).

It must be emphasized that the data reported here are crude, judged by the standards of physical chemistry, and serve to measure only the order of magnitude of the interaction. Precise chemical experiments did not seem warranted at this stage, since our purpose was to compare the chemical results with the bacteriological data, which had already been obtained with commercial oleic

acid, a notoriously impure product, and with Tween 80 manufactured from such material. More precise experiments on the binding of long chain fatty acids by serum albumin are planned.

## SUMMARY

Serum albumin is a protective bacterial growth factor; by binding traces of fatty acid in the media it permits initiation of growth by the smallest possible inocula of tubercle bacilli. Each molecule of albumin binds 3 to 6 molecules of oleic acid (1 to 2 per cent of the weight of the albumin) tightly enough to prevent bacteriostasis, and 9 molecules of oleic acid in equilibrium with a saturated neutral solution. The property requires undenatured albumin. Crystalline  $\beta$ -lactoglobulin has a smaller capacity, and a number of other proteins no perceptible capacity to bind oleic acid.

The inhibitory effect of the commercial product Tween 80 (polyoxyethylene sorbitan monooleate) on the growth of small inocula of tubercle bacilli in liquid media is caused by its content of unesterified oleic acid (0.6 per cent by weight). Purified Tween 80, freed of this contaminating fatty acid, not only permits growth of small inocula, but protects against small amounts of added oleic acid.

The implications of the binding capacity of albumin for its possible physiological significance in the animal body (transport; protection against cytotoxins), and for the structure of the protein, are briefly discussed.

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