THE RELATION OF PROTEIN BINDING TO THE PHARMACOLOGY AND ANTIBACTERIAL ACTIVITY OF PENICILLINS X, G, DIHYDRO F, AND K¹

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It is well recognized that many substances which inhibit bacterial growth *in vitro* may lose a part or all of this antibacterial activity in the presence of human serum. This phenomenon is attributed in part to the fact that the antibacterial agents are adsorbed or otherwise bound to some constituent of serum. The role of the serum proteins in this process has recently been reviewed by Davis (1946). It has been demonstrated by Davis (1942, 1943) and by Gilligan (1943) that the individual sulfonamides are bound to serum albumin to a varying degree, and there is suggestive evidence that the portion of the drug which is bound is inactive against bacteria.

Chow and McKee (1945) have shown by dialysis experiments that crystalline penicillin (G) is bound to serum albumin. Similar results were reported from this laboratory in experiments in which whole serum and crude penicillin were used (McDermott and Nelson, 1945). In the latter experiments, the antibacterial activity of the penicillin-serum complex was not studied. Chow and McKee believed, however, that, although the penicillin was bound to a significant degree, the penicillin-albumin complex was still fully active. In support of this hypothesis is the fact that early studies of penicillin had indicated that human serum had no inhibitory effect upon the action of the drug (Abraham *et al.*, 1941; Rammelkamp and Keefer, 1943).

In contrast to these findings, it was noted by Romansky (1944) and by Bigger (1944) that the presence of serum may interfere with the bioassay of penicillin in the lowest range of the broth dilution methods. Bigger also reported that penicillin is inactivated by incubation in human serum at 37 C. Both of these observations have been repeatedly confirmed in this and in other laboratories, although opinions as to the nature of the phenomena have differed. Additional relevant observations are those of Holmes and Lockwood (1944), who noted the presence of an "antipenicillin factor" in human ascitic fluid and in the serum of humans, horses, and rabbits. The substance responsible for this antipenicillin effect was dialyzable and relatively heat-stable.

Thus, by the end of 1945, evidence had been accumulated which would indicate: (a) that penicillin G would bind to purified albumin and to a constituent of serum which presumably was albumin; and (b) that there was a substance or

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substances in human serum which could destroy or otherwise inactivate penicillin *in vitro*.

Early in 1946 it was first observed that there is a marked discrepancy between the *in vitro* and the *in vivo* (mouse and rabbit) activity of the four penicillins X, G, F, and K (Coghill *et al.*, 1946; Eagle and Musselman, 1946; Hobby *et al.*, 1946). The disparity was striking with penicillin K, which is the most active of the four *in vitro* but is the least active in the treatment of streptococcus infections in mice. In the rabbit, penicillin K is notably less effective in the treatment of syphilis than are penicillins X or G. In man, comparable quantitative observations of the therapeutic effectiveness of penicillin K are not yet available. It was noted, however, in three different laboratories (Coghill *et al.*, 1946; Eagle and Musselman, 1946; McDermott and Tompsett, 1946), that penicillin K apparently disappears from the circulating blood of man with unusual rapidity following parenteral administration in doses of 20,000 to 90,000 units. As the urinary excretion of the penicillin K was notably low in these experiments, it was concluded that the material was destroyed within the body. It was assumed that the apparently rapid disappearance of the penicillin from the blood of man would be responsible for a relatively low therapeutic effectiveness similar to that observed in the mouse and the rabbit.

These two characteristics of penicillin K—a marked difference between its *in vitro* and *in vivo* activity, and a pharmacologic behavior apparently different from closely related compounds—suggested that the phenomenon of protein binding might be operative. Accordingly, an investigation has been conducted of the role of the serum proteins in the pharmacology of penicillin K and the other individual penicillins.

METHODS AND MATERIALS

The method of Hobby (1946) for the assay of penicillin in serum was modified for the purposes of this investigation. The procedure differs from the more commonly used methods in that the increments are smaller, the amount of serum is kept constant in each tube by the addition of normal, pooled human serum, and the inoculum of organisms is small. The details of the method may be seen in table 1. The test organism used is *Streptococcus hemolyticus*, strain C203MV (group A). The end point is read as the first tube in which there is no visible growth after incubation at 37 C for 24 to 30 hours. In experiments in which it was desired to avoid any appreciable amounts of serum in the assay, the same method was employed, except that the dilutions were made entirely in plain broth, and the culture was added in blood broth. The latter contained a suspension of defibrinated rabbit blood in an amount sufficient to give a final concentration of 2 per cent blood. In this type of experiment, the concentrations of penicillin were so chosen that the lowest levels would be in tubes containing not more than 1 to 2 per cent serum. The end point was read as the first tube in which there was no hemolysis or grossly visible growth. The broth was beef heart infusion broth, which contained 1 per cent peptone and 0.5 per cent NaCl, at pH 7.6. The cultures were 18-hour cultures of the organisms noted. The

serum used routinely was pooled human serum heated to 56 C for 30 minutes and filtered through a Seitz filter. Each set of experiments was checked with fresh, sterile unheated serum.

The sensitivity tests were performed in the same way as the standard control in the assay method, and the sensitivity of the test organism was calculated as the concentration present in the tube at the end point. Variations in the final concentration of serum or albumin in the sensitivity tests were attained by appropriate variations in the concentration of these substances in the broth.

The following crystalline penicillins² were used: (1) benzylpenicillin (G); (2) *n*-heptylpenicillin (K); (3) *p*-hydroxybenzylpenicillin (X); and (4) *n*-amylpenicillin (dihydro F).

TABLE 1
Method of assay

DILUTION OF UNKNOWN (FINAL CONCENTRATION OF SERUM = 25 PER CENT)	1:120						1:20						1:4					
Ml of diluted unknown.....	0.2	0.3	0.4	0.5	0.6	0.8	0.2	0.3	0.4	0.5	0.6	0.8	0.2	0.3	0.4	0.5	0.6	0.8
25 per cent pooled human serum in broth.....	0.6	0.5	0.4	0.3	0.2	0	0.6	0.5	0.4	0.3	0.2	0	0.6	0.5	0.4	0.3	0.2	0
Culture.....	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Units per ml in unknown, when sensitivity of organism is 0.01 unit per ml.....	6	4	3	2.5	2.0	1.5	1.0	0.67	0.5	0.42	0.35	0.25	0.2	0.14	0.1	0.083	0.07	0.05

Procedure

I. Three dilutions of unknown serum are made as follows:

(1) a 1:4 dilution in broth;

(2) a 1:5 dilution of (1) using 25 per cent pooled human serum in broth as diluent;

(3) a 1:6 dilution of (2), again diluting with 25 per cent serum.

This gives final dilutions of 1:120, 1:20, and 1:4, each of which is 25 per cent serum.

II. Add the amounts of various dilutions as noted on table.

III. Make up each tube to volume of 0.8 ml with 25 per cent pooled serum.

IV. Add 0.2 ml culture to each tube. (Culture dilution = 2×10^{-6} .)

V. End point is read as first tube showing no growth after 24 to 30 hours' incubation. A standard solution of the same penicillin in pooled serum is assayed as above. The sensitivity of the organism is calculated from this. The readings for any sensitivity are made conveniently by multiplying or dividing the reading given here.

Craig and his associates (1947) have recently demonstrated that significant amounts of as yet unidentified antibiotics were present in a sample of the crystalline sodium salt of penicillin K. Even if it be assumed that all samples of crystalline penicillin K consist of *n*-heptylpenicillin and unknown antibiotics, the biologic and pharmacologic behavior of such crystalline material appears to be sufficiently distinctive to warrant the use of the term K or preferably "K-type" penicillins. In order to simplify the subsequent discussion, the term K is used to designate crystalline material which consists of *n*-heptylpenicillin and possibly significant amounts of as yet unidentified "penicillins."

In the dialysis experiments, a cellophane bag, which contained 5 ml of pooled human serum or protein solution, was suspended in 20 to 100 ml of phosphate

² The penicillins G, K, and dihydro F were obtained from Chas. Pfizer and Company. The penicillin X was obtained from Lederle Laboratories.

buffer (pH 7.38) containing 2 to 5 units of penicillin per ml. Dialysis was conducted for 48 hours at 10 C or for 4 hours in a shaking machine at room temperature. In some experiments, a second bag of serum containing 10 or 20 units per ml of the same penicillin was added to the flask in order to determine that equilibrium had been reached. It was assumed that this had occurred if the penicillin concentrations in the two bags were the same at the end of the experiment. A further check was made by determining the time at which the concentration inside the bag stopped rising.

RESULTS

Effects of serum on the sensitivity of Streptococcus hemolyticus to individual penicillins. A series of sensitivity tests were performed with the standard test organism C203MV in media which contained various concentrations of pooled human serum. The basic medium was 2 per cent blood broth, and the final concentrations of serum were 10, 20, and 30 per cent, respectively. The concentration of penicillin required to prevent growth of the organisms in 2 per cent blood broth was compared with the concentration required in the presence of 10 to 30 per cent serum. The percentage of efficiency of the penicillin was then calculated as follows:

$$\frac{\text{Sensitivity of organism in 2 per cent blood broth}}{\text{Sensitivity in 2 per cent blood broth plus serum}} \times 100$$

The calculation was made to eliminate the day to day variations which always occur in a bioassay, to make the values comparable on either a unitage or a gravimetric basis, and to eliminate differences in the sensitivity of the particular organism to the individual penicillins. Thus a decrease in percentage of efficiency represents a decrease in the penicillin sensitivity of the organism, i.e., an increase in the concentration of penicillin necessary for the prevention of growth. As may be seen in figure 1, with the addition of increasing amounts of serum to the medium, there is a progressive decrease in the sensitivity of the test organism. The changes produced by the addition of serum are of the same degree for penicillins X, G, and dihydro F. The average efficiency of these three penicillins in 30 per cent serum was 36, 41, and 43 per cent, respectively. In contrast, the effect of serum to penicillin K was of an entirely different magnitude. The average efficiency of penicillin K in 30 per cent serum was only 7 per cent.

In analysis of the serum effect, it is necessary to consider three factors which might influence the results: (1) enhancement or inhibition of growth of the organism by the serum; (2) destruction of the penicillin by the serum during the incubation necessary for the bioassay; and (3) inactivation of the penicillin by binding to some constituent of the serum. Accordingly, the possible operation of these factors was investigated.

Effect of serum on the organism. The highest concentration of serum used in the sensitivity tests was 30 per cent. Higher concentrations of serum are known to exert an inhibitory effect upon the growth of the particular test

organism used (Elias *et al.*, 1945). As may be seen in figure 2, however, the growth curves observed after the use of large and small inocula of C203MV were identical in blood broth and in the concentrations of serum which were used in the present experiments. In control experiments, it was established that the rise in pH produced by the addition of serum did not alter the results.

FIG. 1. EFFECT OF SERUM ON EFFICIENCY OF FOUR PENICILLINS

Destruction of penicillin by incubation in serum. Known amounts of the crystalline penicillins were added to serum, and the mixture was incubated at 37 C for 24 hours. Aliquots were removed for assay immediately after addition of the penicillin and at intervals thereafter. All samples were stored at -20 C until the completion of an experiment and were then assayed at the same time. Most of the experiments were performed with penicillins G and K.

Fresh human serum was collected from 4 to 6 persons, pooled, and used immediately. Parallel experiments were made with this and with a portion of the

same serum heated at 56 C for $\frac{1}{2}$ hour. In addition, heated, pooled human serum which had been filtered through a Seitz filter was used.

FIG. 2. GROWTH OF *STREPTOCOCCUS HEMOLYTICUS* (C203MV) IN THE TEST MEDIA

TABLE 2
Incubation of penicillins G and K in human serum

PENICILLIN	HOURS OF INCUBATION AT 37 C	PER CENT RESIDUAL		
		Average*	Minimum	Maximum
G	2	95	50	100
	4	92	50	100
	6	72	44	100
	12	35	25	65
	24	7	3	12.5
K	2	92	67	100
	4	91	65	100
	6	53	32	67
	12	29	25	33
	24		<3	4

* These values represent 12 experiments with G and 8 with K.

In the majority of the experiments, the initial concentration of penicillin was 10 units per ml of 98 to 99 per cent serum. In other experiments, 0.2 to 0.5

units of penicillin per ml were incubated in 98, 30, 20, and 10 per cent serum, which was diluted in the broth used in the sensitivity test. The results may be summarized as follows (table 2): (1) Penicillins G and K, are inactivated under the conditions described. (2) Both are inactivated from 90 to 100 per cent in 24 hours by whole serum, whether it be heated or unheated.

The inactivation does not appear to be a first-order reaction. With rare exceptions, no inactivation was apparent after 2 hours' incubation. Inactivation usually appeared after 4 to 6 hours' incubation of penicillin G and after 2 to 4 hours' incubation of penicillin K. Once their action appeared, it proceeded at a fairly regular rate throughout the 24-hour period. It should be emphasized

TABLE 3
Results of a typical dialysis experiment

PENICILLIN	SERUM UNITS PER ML	DIALYZATE UNITS PER ML
X	2.5	1.5
G	2.5	1.0
Dihydro F	4.0	1.3
K	15.0	0.5

TABLE 4
Binding of penicillin to human serum and purified plasma fractions

PENICILLIN	AVERAGE PER CENT BOUND				
	Human serum	Human albumin Fraction V	Bovine albumin	Human plasma Fractions I, II, III, IV-1, IV-1,1, IV-4	Bovine plasma Fraction II
X	47		53		0
G	58	60	52	0	0
Dihydro F	63		66		0
K	91	86	86	0	0

that in this type of experiment the behavior of the same preparations of penicillins G and K varies widely from day to day. In any individual experiment, however, in which penicillins G and K were compared simultaneously under identical conditions, the onset of inactivation consistently appeared earlier with penicillin K than with penicillin G. It was not possible to demonstrate that the rate or the onset of inactivation of penicillins G and K was any different in heated than in unheated serum. In the experiments in which low concentrations of penicillins G and K (0.2 to 0.5 units per ml) were incubated with 10, 20, and 30 per cent serum in broth, no inactivation of the penicillins appeared during a 12-hour period of observation. The concentrations of penicillins and serum used in the latter experiments are comparable to those usually present during the bioassay of specimens of serum obtained from a penicillin-treated subject.

Binding of penicillin to serum. The results of the dialysis experiments may be seen in tables 3 and 4. Each value in table 4 represents the average of

6 to 8 dialysis experiments with the particular penicillin. The variation between individual experiments was slight. As may be seen, there was a significant degree of binding of all of the penicillins to some constituent of serum. Penicillin X was bound the least (47 per cent). Penicillins G and dihydro F were bound to a higher degree, i.e., 58 and 63 per cent, respectively. In contrast to these values, the degree of binding of penicillin K was 91 per cent, or almost

FIG. 3. EFFECT OF ALBUMIN ON EFFICIENCY OF FOUR PENICILLINS

double the average value for penicillin X. As may also be seen in table 4, in each instance the degree of binding of an individual penicillin to serum and to a solution of 5 per cent bovine albumin was of the same order of magnitude. The binding of all four penicillins to bovine globulin (fraction II)³ was investigated. In addition, penicillins G and K were dialyzed against fractions I,

³ The bovine globulin was obtained through the courtesy of Mr. E. B. Lesh, Armour Laboratories, Chicago, Illinois.

II, III, IV-1, IV-1, 1, IV-4, and V of human plasma.⁴ The concentrations of the individual protein fractions approximated those usually present in plasma. As may be seen (table 4), the degree of binding of penicillins G and K to human fraction V closely approximated the degree of binding of these penicillins to bovine albumin and human serum. In contrast, neither penicillin was bound to any of the other protein fractions of plasma.

Effect of albumin on the sensitivity test. The effect of bovine albumin on the sensitivity of hemolytic streptococci to the four penicillins was investigated. The concentrations of albumin in the media were comparable to those attained by the addition of 10, 20, and 30 per cent serum. As may be seen in figure 3,

FIG. 4. GROWTH OF STREPTOCOCCUS HEMOLYTICUS (C203MV) IN THE TEST MEDIA

the effect of bovine albumin on the efficiency of the penicillin was similar to that of human serum. As with serum, the albumin effect was most pronounced with penicillin K, but was significant with all of the penicillins tested. The factor of pH was controlled as in the previous experiments. The addition of 1.0 per cent of bovine albumin did not alter the growth curve of the organism (figure 4). Comparison of the data in figures 1 and 3 indicates that, although the effect of albumin parallels that of serum, the latter produces an effect of greater magnitude. This suggests that other factors may also be present.

⁴ The fractions of human plasma were obtained through the courtesy of Dr. S. Howard Armstrong, Jr., Harvard University Medical College.

Sensitivity tests with pneumococcus and Staphylococcus aureus. In order to exclude the possibility that the results observed were caused by factors unique to the particular test organism used, similar experiments were performed with other organisms. *Pneumococcus* type II (D-39) and *Staphylococcus aureus* (Oxford H strain) were the organisms tested. The results are presented in table 5. As may be seen, with these organisms also, the presence of serum decreased the efficiency of all of the penicillins, and the effect was most pronounced with penicillin K.

TABLE 5

Comparative efficiency of penicillins against Staphylococcus and pneumococcus

TEST ORGANISM	PENICILLIN	EFFICIENCY OF PENICILLIN IN 30 PER CENT SERUM
<i>Staphylococcus aureus</i>	X	% 67
	G	57
	K	5
<i>Pneumococcus</i> type II	X	39
	G	40
	K	9

DISCUSSION

On the basis of these observations, it appears that (1) the antibacterial activities of four penicillins were antagonized *in vitro* by serum and by the albumin fraction of serum; (2) the degrees of antagonism were quantitatively different for the individual penicillins; and (3) among these four penicillins, the degrees of reduction in antibacterial activity caused by serum and albumin were roughly in direct proportion to the degrees of binding to these substances as demonstrated by dialysis.

The most satisfactory rationalization of these data is that the individual penicillins bind *in vitro* to a varying degree to the albumin component of serum, and that the resulting penicillin-albumin complex has little or no antibacterial activity. Investigation of alternative explanations for the observed phenomena have been made. The growth curve of the test organism in 2 per cent blood broth was not altered by the addition of 10 to 30 per cent serum or 1.0 per cent bovine albumin (figures 2 and 4). The possibility that serum or albumin may increase the penicillin resistance of bacteria without affecting their growth characteristics cannot, however, be explicitly excluded.

Although inactivation of the penicillins occurred after prolonged incubation in serum, it does not appear that this reaction is an explanation for the decreased activity of these drugs in the presence of serum or albumin. This type of inactivation during incubation did not usually start until after 2 to 4 hours' incubation, a time at which much of the effect of the penicillin upon the organisms would presumably have already occurred. Moreover, inactivation of penicillin re-

sulting from incubation in serum was notably irregular, in contrast to the serum effect upon the penicillin sensitivity of the organisms, which was regular and readily reproducible. The uniformity of the antagonistic effect of serum on an individual penicillin, coupled with the marked difference among the different penicillins, makes it seem unlikely that the effect is a result of destruction of penicillin by the incubation during the bioassay procedure.

It should be noted that important differences exist between the conditions which obtain *in vivo* and the conditions of penicillin-serum incubation experiments such as those presented above. The stability of all of the penicillins is considerably different at various levels of pH (Benedict *et al.*, 1946), and the pH of human serum *in vitro* is 8.0 or higher. Moreover, the delay in the onset of inactivation which occurs on incubation *in vitro* strongly suggests that the inactivating substance is not normally present in serum but is produced artificially by the procedure.

If protein-bound penicillin is inactive, it would be anticipated that a penicillin that is bound to a high degree would be considerably less efficient in the presence of serum or albumin than a penicillin that is bound to a lesser degree. In the present experiments, penicillin K, approximately 90 per cent of which is bound to serum or albumin, lost 85 to 90 per cent of its activity *in vitro* against the test organism in the presence of 30 per cent serum or 1.5 per cent albumin. In contrast, under the same conditions penicillin X, which binds much less (47 per cent), lost only 40 to 60 per cent of its activity.

It has not been possible, as yet, to determine by direct observation whether the penicillin-albumin complex exerts antibacterial action *in vitro* or *in vivo*. The reason for this is that the degree of binding of penicillin to albumin, like that demonstrated for other drugs (Davis, 1946), presumably represents a balance between the concentrations of bound and unbound drug present in the system. The degree of this type of binding is readily altered by dilution. Therefore, measurement of the activity of the penicillin-albumin complex by bioassay or after parenteral administration, both of which necessarily involve dilution, cannot represent direct observation of the activity of the original material before dilution.

Despite the absence of direct proof, however, it would seem that the protein-bound portion of the penicillin is inactive *in vitro*, from the facts that each penicillin is bound to albumin or serum to a certain degree; that the degree of binding and the loss of antibacterial activity in protein solutions are constant and are unique for each penicillin; and that the loss of antibacterial activity in protein solutions does not appear to result from penicillin destruction or from action of the protein on bacterial growth.

Whether penicillin is bound to albumin *in vivo* to the same extent as occurs *in vitro* has not been determined for the reasons noted previously. There is evidence which suggests, however, that the binding of penicillin to protein *in vivo* does occur. There is a striking parallelism between the efficiency of a particular penicillin when tested *in vitro* in the presence of albumin and its therapeutic effectiveness *in vivo*. The *in vitro* activity of penicillin X is the least

affected by serum or albumin, and the drug binds to albumin to the least degree of any of the penicillins tested. In experimental infections *in vivo*, penicillin X is the most active of these four penicillins (Eagle and Musselman, 1946; Hobby *et al.*, 1946). In contrast, the *in vitro* activity of penicillin K is considerably reduced by serum and albumin, the drug binds to albumin to a high degree and is the least active of these four penicillins when tested *in vivo*. Thus the apparent disparity between the *in vitro* and the *in vivo* activity of these four penicillins disappears when the *in vitro* testing is performed in the presence of serum or albumin.

These results are essentially the same as the results previously observed in similar experiments with the sulfonamides. In both instances, all of the compounds studied were bound to albumin to a significant degree. With the sulfonamide drugs, it was reported (Davis and Wood, 1942) that the degree of binding parallels the *in vitro* activity when the latter is determined in the absence of protein. In the present investigation, a similar relationship was observed. In the absence of protein, penicillin X is the least, and penicillin K the most, active of the four compounds.

If the penicillins are bound to the plasma albumin *in vivo*, it would be anticipated from the experience with other albumin-bound agents that the degree of binding of a particular penicillin would exert a considerable influence on its distribution, conservation, or excretion by the kidneys, and ultimate fate. Moreover, as suggested for the sulfonamides by Davis (1943, 1946), appreciably lower concentrations of penicillin would be necessary for therapeutic effectiveness in fluids which are poor in protein, such as the cerebrospinal fluid, than would be required in the blood or the extracellular fluid in general. Possible support for the latter notion is afforded by the excellent results which have been observed in the treatment of neurosyphilis following the administration of penicillin solely by the intramuscular route. On the regimens which have been therapeutically successful, only minute amounts of penicillin appear in the cerebrospinal fluid even after 2 weeks of continuous therapy. The penicillin concentrations in the interstitial fluid of the brain are presumably equally low, for both fluids eventually attain equilibrium. Despite the low concentrations of penicillin attained in these protein-poor fluids, the therapeutic action of the drug is striking.

Regardless of the occurrence of binding to protein *in vivo*, the effects observed are important in studies of the absorption and distribution of the individual penicillins in man. The numerous disadvantages of the bioassay of penicillin are greatly multiplied by lack of satisfactory controls. The phenomena observed here interfere with the bioassay of all four penicillins in specimens of serum, and the effect is quantitatively different in each.

The conventional bioassay consists of (1) a stepwise dilution of the unknown specimen into a series of tubes, and (2) the addition of broth and culture to the tubes, bringing all tubes to a constant volume. The unknown specimen is then compared with a specimen of known concentration, and the penicillin sensitivity of the organism is calculated from this standard. A direct calculation is then made from standard to unknown, using only the dilution of the penicillin in the calculation.

As we have seen, the penicillin sensitivity is different in different concentrations of serum. As each tube in the lower range of the test contains a different concentration of serum, the penicillin sensitivity is different in each tube. Consequently, an end point in the lower range is directly comparable *only* with a standard which contains the same concentration of serum, and is not directly comparable with another concentration of penicillin in serum, or with any standard which does not contain serum. It should be emphasized that this factor is not controlled simply by starting with a standard which is in serum. To correct this error, one may maintain a constant percentage of serum in each tube by the addition of pooled serum. This allows a direct comparison of unknown to standard. By this method, using *Streptococcus hemolyticus* C203MV as the test organism, the lowest concentration of the four penicillins which can be measured in an unknown serum varies from 0.05 unit per ml of penicillin X to 0.4 to 0.5 unit per ml of penicillin K.

Following the parenteral administration to an adult of 20,000 to 90,000 units of any of the four penicillins used in the present study, the concentration in the serum would usually fall below 0.3 unit per ml during the first hour after injection of the drug. As it is impossible to measure penicillin K at this concentration in serum, the false impression would be created that the penicillin K had "disappeared" from the blood within 30 to 60 minutes. In contrast, penicillins G and X, which can be measured in appreciably lower concentrations, would be detectable for much longer periods of time after the parenteral injection.

It appears, therefore, that the rapid "disappearance" of penicillin K from the blood of humans, noted in this and in other laboratories, is in all likelihood an artifact attributable to the marked effects of serum (presumably albumin binding) upon the activity of penicillin K in the *in vitro* assay. It should not be inferred from these observations that the stability of penicillin K within the body is necessarily the same as that of the other penicillins. On the contrary, if the same high degree of binding to albumin occurs *in vivo* as *in vitro*, it is conceivable that the rate of excretion through the kidney could be sufficiently slowed and the distribution in the tissues altered so that more of this penicillin per dose would be exposed to destructive influences within the body. It might be possible, by the use of much larger doses than have hitherto been employed, to obtain a more accurate estimation of the comparative rates of disappearance of the individual penicillins from the circulating blood. An investigation of this aspect of the subject is in progress.

As the effect of serum upon the sensitivity of the bioassay may vary so considerably among individual penicillins, the magnitude of the effect for each newly introduced penicillin must be determined before its distribution within the body can be properly studied. Moreover, the apparent correlation between the binding of penicillin to albumin *in vitro* and the therapeutic effectiveness of the drug *in vivo* suggests that this procedure should also be applied to all new penicillins.

SUMMARY

It was observed that the antibacterial activities of four penicillins, X, G, dihydro F, and K, were antagonized *in vitro* by serum and by the albumin fraction of serum.

The degrees of antagonism were quantitatively different for the individual penicillins.

Among these four penicillins, the degrees of reduction in antibacterial activity caused by serum and albumin were roughly in direct proportion to the degrees of binding to these substances as demonstrated by dialysis. Penicillin X, which was 47 per cent bound, lost 40 to 60 per cent of its activity in the presence of 30 per cent serum. Penicillin K, which was 91 per cent bound, lost 85 to 90 per cent of its activity when similarly tested.

There is an inverse relationship between the degree of binding of the individual penicillins to serum *in vitro* and the reported effectiveness of these penicillins in the treatment of infections in experimental animals.

ADDENDUM

Subsequent to the preparation of this manuscript, a study of the distribution of penicillins G and K after intravenous administration in dogs was reported by Richardson and his associates (Proc. Soc. Exptl. Biol. Med., 1946, 63, 514). Although different techniques were employed, certain observations were made which are in agreement with those reported here. These investigators observed that in the cup test assay it was not possible to obtain complete recoveries of penicillins G and K added to plasma. The recovery of K was considerably lower than that of G. Evidence was presented that the low recovery was not due to destruction of the penicillin. It was also observed in dialysis experiments that penicillin K was bound by plasma to a higher degree than penicillin G.

Thus it would appear that, insofar as they are parallel, the observations reported here confirm those of Richardson and his coworkers.

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