general circulation.

Summary. Sodium nitrite ($NaNO_2$), isomannide dinitrate (I2N), glyceryl trinitrate (G3N), erythrityl tetranitrate (E4N) and mannityl hexanitrate (M6N) injected intravenously in doses of 1 mg/kg into the cat anesthetized with pentobarbital had effects on the mean carotid blood pressure which did not differ significantly in magnitude and duration.

$NaNO_2$ appeared to have an immediate and powerful dilating action on the venous system while E4N may have had a similar but weaker action. I2N, G3N and M6N appeared not to affect the venous system significantly.

Dr. J. C. Krantz, Jr., of the University of Maryland Medical School, kindly supplied the isomannide dinitrate.

15898

Influence of Protein-Binding on the Interpretation of Penicillin Activity *In vivo*.*

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Introduction. In evaluation of the therapeutic use of penicillin and other antibacterial agents, considerable interest was centered on observations of the height and duration of the serum concentrations attained after administration to humans. Studies of this type have been of value in determining the dosage of penicillin, the frequency and route of administration, and the influence of various vehicles and adjuvants on absorption and excretion.

The availability of the individual purified penicillins, X, G, F, K and dihydro F, has stimulated renewed interest in this type of investigation. It has been reported that penicillin X affords higher and more prolonged concentrations in serum than an equal dose of penicillin G.[1,2] Likewise penicillin X has been found to be significantly more effective than penicillin G in the treatment of infections in experimental animals.[3,4]

Of particular interest have been the studies of penicillin K. *In vitro,* penicillin K is distinctly more active than penicillins X, G, F and dihydro F. In the treatment of infections in experimental animals, however, penicillin K has been found to be the least effective of these 5 penicillins.[3,4] It was observed simultaneously in 3 laboratories[5–7] that, following intramuscular injection, penicillin K apparently disappeared rapidly from the circulating blood. It was also observed that the total urinary excretion of penicillin K was low, and it was concluded that the drug was ineffective because of rapid destruction in the body.[5,6]

Eagle[8] has reported a possible explanation of the differences in pharmacologic behavior among the individual penicillins. In a study of penicillins X, G, F and K, he observed that all 4 penicillins were slowly inactivated

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1 Ory, E. M., Meads, M., and Finland, M., *J. A. M. A.*, 1945, **129**, 157.

2 Eagle, H., *J. Exp. Med.*, 1947, **85**, 163.

3 Hobby, G. L., Burkhart, B., and Hyman, B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 296.

4 Eagle, H., *J. Exp. Med.*, 1947, **85**, 175.

5 Eagle, H., and Musselman, A., *Science,* 1946, **103**, 618.

6 Coghill, R. D., Osterberg, A. E., and Hazel, G. R., *Science,* 1946, **103**, 709.

7 McDermott, W., and Tompsett, R., cited in *J. A. M. A.*, 1946, **131**, 271.

8 Eagle, H., *J. Exp. Med.*, 1947, **85**, 141.

by a thermostable component of serum. Penicillin X was the least susceptible of the 4 to this inactivation. In addition, he reported that penicillin K was rapidly inactivated by a thermolabile component of serum, and that the rate of this latter type of inactivation increased as the concentration of penicillin decreased. Eagle postulated that the apparent differences in the periods during which the individual penicillins were present in the circulating blood could be explained on the basis of the relative susceptibilities of these penicillins to inactivation by serum *in vitro*.

In contrast to previous observations on penicillin K, Richardson and his co-workers[9] have recently reported that from a given plasma concentration the rate of disappearance of penicillin K is the same as that of penicillin G. These investigators found in dogs that after the intravenous administration of penicillins G and K the penicillin K was localized in the liver in higher concentrations than penicillin G. They further reported that both penicillins appeared to be bound by plasma but that penicillin K was bound to a greater degree than penicillin G. Evidence was obtained that the low recovery (activity) of penicillin K in plasma was not due to destruction of the penicillin.

In a previous communication from this laboratory,[10] it was reported that the antibacterial activities of penicillins X, G, dihydro F and K were antagonized by serum and the albumin fraction of serum. The degrees of antagonism were quantitatively different for the individual penicillins. Among these 4 penicillins, the degrees of reduction in antibacterial activity caused by serum and by albumin were roughly in direct proportion to the degrees of binding to these substances demonstrable by dialysis. Penicillin X, which was bound 47%, lost 40 to 60% of its *in vitro* activity in the presence of serum or albumin. Penicillin K was bound approximately 90%, and when similarly tested lost 85 to 90% of its activity. The degrees of binding and the degrees of reduction in activity of penicillins G and dihydro F in serum were intermediate between the values for penicillins X and K. Moreover, the reduction in antibacterial activity was independent of any actual destruction of penicillin by the serum, and was the same in fresh serum as in serum heated at 56°C for 30 minutes.

These data indicate that the antibacterial activity of the individual penicillins is exerted only by the unbound portion of the drug. The observations afforded a possible explanation of the apparent discrepancies between the *in vitro* and *in vivo* activities of the 4 penicillins studied. When the *in vitro* testing was performed in the presence of serum or the albumin fraction of serum, the relative activities of penicillins X, G, dihydro F and K were the same *in vitro* as *in vivo*.

In addition it was reported that the phenomena observed interfere with the bioassay of penicillin in serum, and the interference is quantitatively different for each penicillin. The measurement of penicillin in serum by a tube dilution method depends on the determination in a standard control system of the sensitivity of a test organism to penicillin. From this value, the penicillin concentration in an unknown specimen is calculated according to the following formula:

$$\text{units per cc in unknown} = \frac{\text{sensitivity of test organism (u/cc)}}{\text{reciprocal of the highest dilution of unknown specimen which inhibits growth}}$$

For this calculation to be valid, *the sensitivity of the test organism must be constant throughout every dilution of the bioassay procedure*. It was observed, however, that the values for the sensitivity of 2 common test organisms — *Streptococcus hemolyticus* (C203MV) and *Staphylococcus aureus* (Oxford H)—vary in different concentrations of serum, an effect which is presumably due to binding of the penicillin to albumin. The differences in sensitivity were thus quantitatively different among the individual peni-

9 Richardson, A. P., Miller, I., Schumacher, C., Jambor, W., Pansy, F., and Lapedes, D., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 514.

10 Tompsett, R., Shultz, S., and McDermott, W., *J. Bact.*, 1947, **53**, 581.

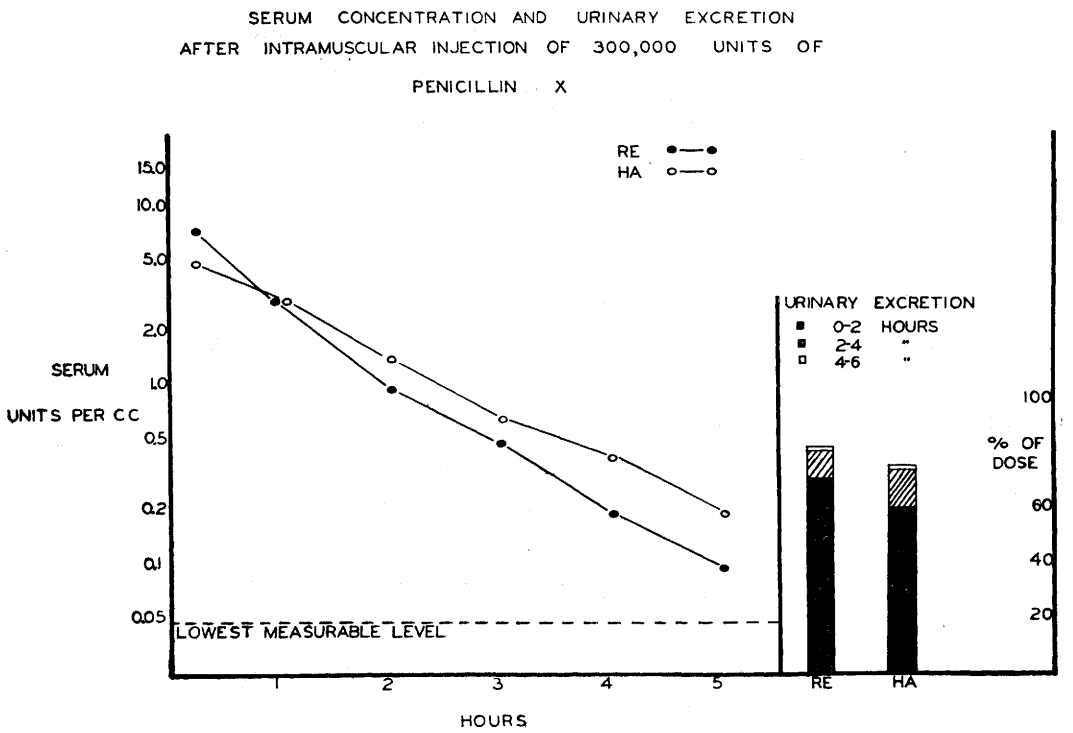

FIG. 1.

cillins and were most marked with penicillin K. A method of bioassay was developed in which the effect of serum was kept constant by maintaining a constant concentration in each tube through the addition of pooled serum. By this method, using *Streptococcus hemolyticus* C203MV as the test organism, the lowest concentration of the 4 penicillins measurable in serum varied from 0.05 unit per cc of penicillin X to 0.4 to 0.5 unit per cc of penicillin K.

These observations suggested that the findings of prolonged serum concentrations of penicillin X, and the rapid "disappearance" of penicillin K from the blood might be the result of failure to consider the effects of serum on the bioassay itself. Accordingly it seemed advisable to evaluate the absorption, excretion and comparative rates of disappearance in the light of the differences in the degrees to which they are bound by serum.

Experimental. Methods and Materials. Four crystalline penicillins† were used: (1) *p*-hydroxybenzylpenicillin (X), (2) benzylpenicillin (G), (3) *n*-amylpenicillin (dihydro F), and (4) *n*-heptylpenicillin (K).‡

The penicillins were administered intramuscularly to healthy adults in single doses dissolved in normal saline solution. Assays of penicillin in serum were performed by the method previously described.[10] The test organism was *Streptococcus hemolyticus* C203-MV. Measurements of penicillin in urine were performed by a similar tube dilution method. A few of the urine samples were

† The penicillins G, dihydro F and K were supplied by Charles Pfizer and Co. The penicillin X was obtained from Lederle Laboratories.

‡ The sample of penicillin K was a crystalline sodium salt which by the method of Craig and his associates[11] was shown to contain approximately 80% of n-heptylpenicillin, and 15% or more of other as yet unidentified antibiotics. Although this material did not consist entirely of n-heptylpenicillin the biologic and pharmacologic behavior appears to be sufficiently distinctive to warrant the use of the term K or "K-type" penicillin.

11 Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and duVigneaud, V., *J. Biol. Chem.*, in press.

SERUM CONCENTRATION AND URINARY EXCRETION AFTER INTRAMUSCULAR INJECTION OF 300,000 UNITS OF PENICILLIN G

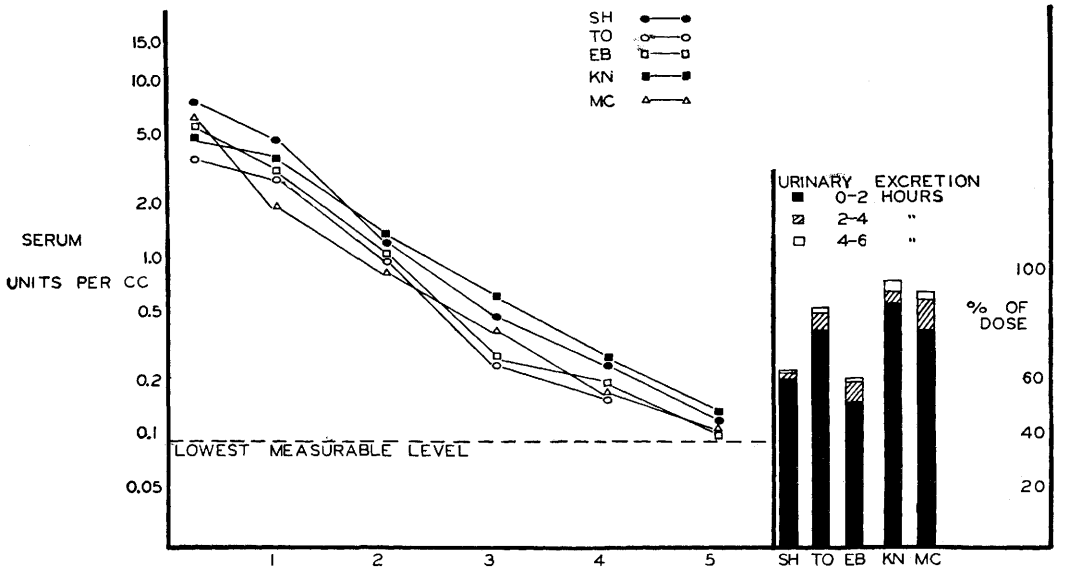

FIG. 2.

SERUM CONCENTRATION AND URINARY EXCRETION AFTER INTRAMUSCULAR INJECTION OF 300,000 UNITS OF PENICILLIN DIHYDRO F

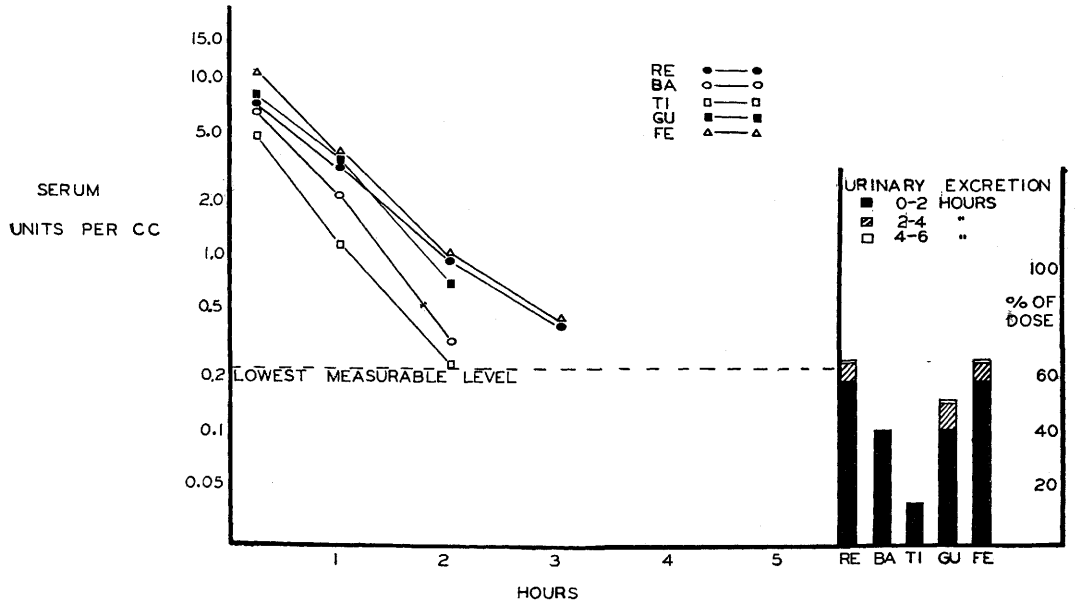

FIG. 3.

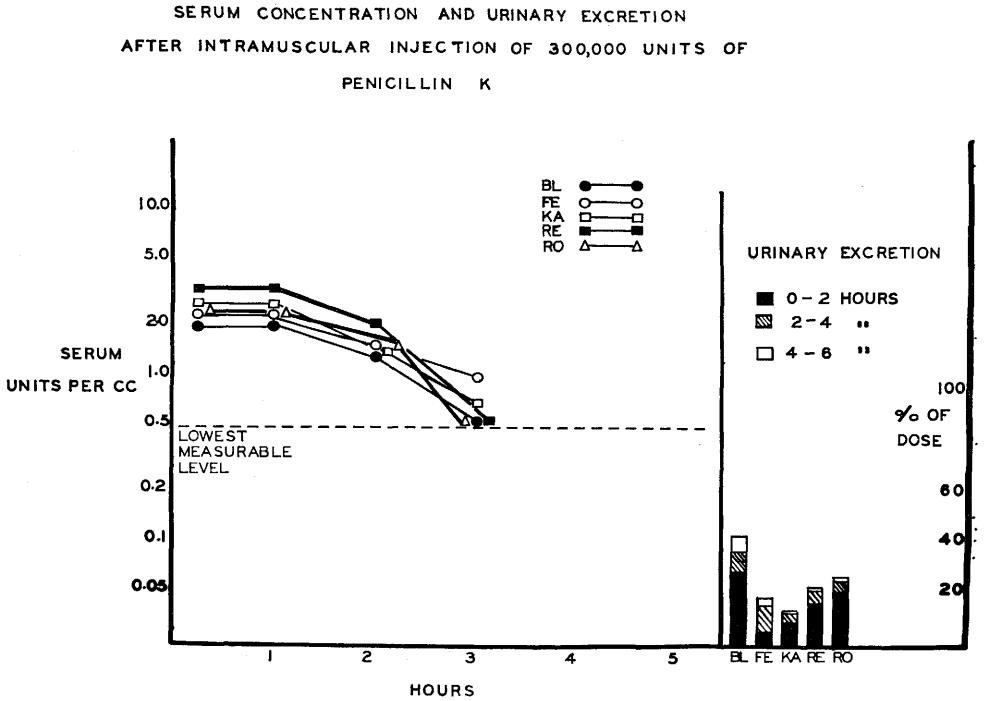

FIG. 4.

also assayed by the standard Oxford cup plate method.

Results. In Fig. 1 to 4 may be seen the serum concentrations of the 4 penicillins attained in the individual subjects after single doses of 300,000 units. The serum concentrations of penicillin X (Fig. 1) in the 2 subjects tested were highest at 15 minutes and fell at a regular rate throughout the test period. In both instances, penicillin X was present in concentrations well above the lowest measurable concentration at the end of the experiment. In Fig. 2 may be seen the values obtained in 5 subjects who received penicillin G. The results were essentially identical with those obtained with penicillin X. The range of values among the different subjects at each time period was small. At the end of 5 hours, the serum concentrations of penicillin G in 4 of the 5 subjects were still measurable.

In Fig. 3 are shown the results of a similar experiment with penicillin dihydro F. With this penicillin the effect of serum in the assay was more noticeable. When the concentration of serum was properly controlled the lowest measurable level of penicillin dihydro F was 0.25 unit per cc. In 3 of 5 subjects, the serum concentration had fallen below this level at the end of the 3rd hour, and in the other 2 at the end of the 4th hour.

In Fig. 4 may be seen the values obtained with penicillin K. The values in the individual subjects were remarkably uniform. Fifteen minutes after injection the levels were not so high as were observed with the other penicillins. One hour after administration, however, the penicillin concentrations were at the same level as had been present at 15 minutes. Moreover, at one hour, the height of the concentrations of penicillin K was essentially the same as was seen at the same interval after the administration of the other penicillins. It is evident that the rate of fall in serum concentrations of penicillin K was not rapid as compared with the other penicillins. A comparison of Fig. 4 with Fig. 1 and 2 discloses that in every instance the concentrations of penicillins X and G at the end of 4 hours were well below the lowest level at which penicillin K can be detected (0.5 unit per cc). Thus it is impossible to

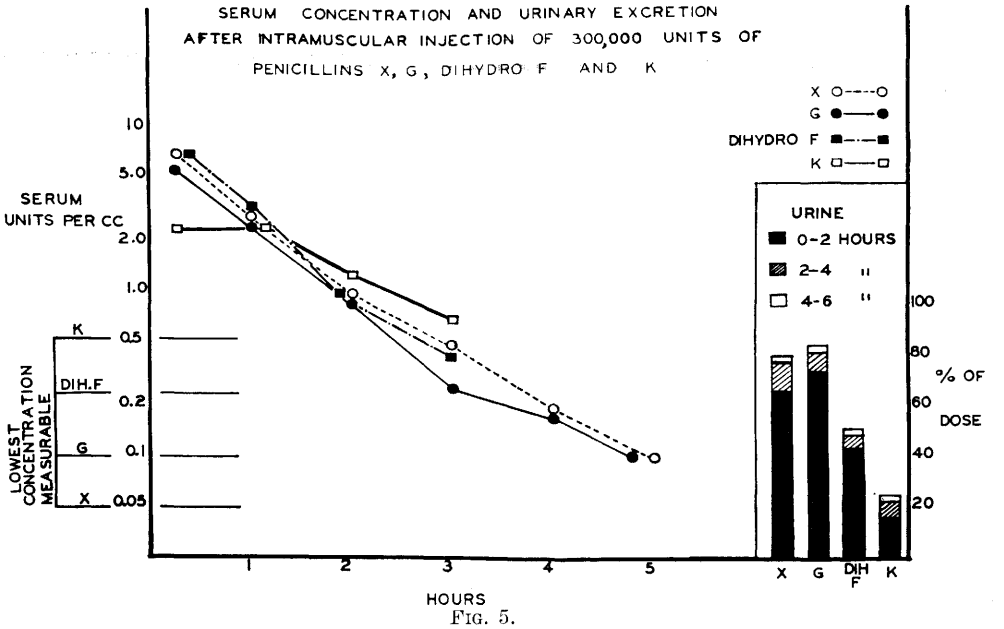

FIG. 5.

establish whether penicillin K was also present in the same concentrations as the other penicillins at the end of the 4th hour after injection. From the form of the curves, however, it is highly probable that the disappearance of penicillin K after the 3rd hour is only a reflection of the limitation of the bioassay of penicillin K.

The similarity of the levels of the 4 penicillins may be illustrated by the results in a single subject. In Fig. 5 are presented the values obtained with the 4 penicillins in a single representative subject. *As may be seen during the first 3 hours, when all the penicillins were present in measurable concentrations, there was no difference among the 4, except for the lower peak of penicillin K at 15 minutes.* In this subject the concentration of penicillin K 3 hours after injection was actually slightly higher than was observed at the same interval after the administration of the other 3 penicillins. (The mean serum concentration of penicillin K in the 5 subjects 3 hours after injection was also higher than the mean values obtained with the other penicillins). Four or 5 hours after injection the concentrations of penicillins X and G although low were above the minimum detectable level for these particular penicillins.

The influence of the antagonistic action of serum on the interpretation of the values obtained with penicillin K may also be demonstrated by a comparison of 2 methods of assay. In Fig. 6 may be seen the serum concentrations of penicillin K in one of the subjects as measured simultaneously by 2 methods. The solid line represents the values obtained with the method described above. The broken line represents values obtained when the same sera were assayed by a standard dilution method in which no correction is made for the influence of serum in the individual dilutions of the assay. The latter curve is similar to those previously reported in studies of penicillin K.[5,6] As may be seen in Fig. 6, when high concentrations are present, the values obtained by the 2 methods are nearly the same. As the concentration in the serum falls, however, the difference becomes increasingly greater. The lower the actual concentration of penicillin becomes, the more of the unknown serum will be present in the tube in which the endpoint occurs. Therefore, as the concentration of penicillin decreases the error in this type of assay is magnified as a result of failure to consider that the antagonistic action of serum at the endpoint has been increased. Thus, failure to consider these opposing factors in this experiment with penicillin K caused the

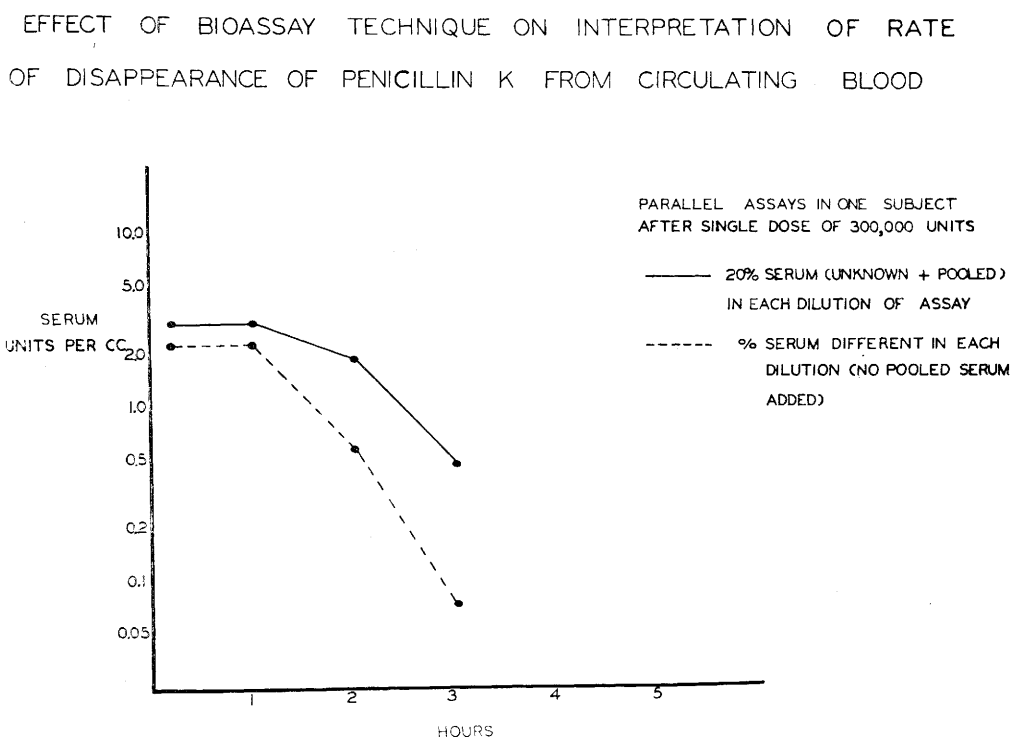

FIG. 6.

rate of fall in serum concentration to appear rapid, and *increasingly more rapid as the actual concentration became lower.*

Although the data which were obtained with penicillin X are too few to be conclusive, it is of interest to compare the values for serum concentrations of penicillins X and G as presented in Fig. 1 and 2. As may be seen there was no significant difference in the serum levels of these 2 penicillins during the course of the experiment. At the end of 5 hours, however, in 4 of the 5 subjects who received penicillin G, the serum concentrations were just above the lowest measurable level. If the experiment had been carried out for a longer period, it is likely that penicillin X could have been measured for a longer time than penicillin G.

Similar studies were carried out with doses of 20,000 units. The serum concentrations attained in the individual subjects are shown in Table I. The general distribution of the values was the same with all 4 penicillins. As in the previous experiments during the time in which all 4 penicillins were measurable, there was no great difference in concentration among the individual penicillins. After 30 to 60 minutes, with only one exception, the concentrations of penicillin X, G and dihydro F fell to levels which were well below the lowest level at which penicillin K can be measured.

In Fig. 1 to 4 are also presented the cumulative urinary excretions of the 4 penicillins for a 6-hour period after administration. The average excretion of penicillin X was 82%; penicillin G, 84.6%; penicillin dihydro F, 51.4%; and penicillin K, 24%. These results are in general agreement with those previously reported.[6,7] From other studies of substances bound to a high degree by plasma protein, it might be anticipated that the degree of binding of penicillin K would affect its rate of excretion in the urine. In Table II may be seen the 6-hour excretion of the 4 penicillins, expressed in terms of percentage of the total excreted in each 2-hour period. Despite considerable variation from one subject to another, it is apparent that the pattern of excretion of penicillin K is

TABLE I.
Serum Concentrations (Units per cc) of Penicillins X, G, Dihydro K and F After Intramuscular Injection of 20,000 Units.

Penicillin	Lowest measurable levels in serum controls units per cc	Minutes after administration 15	30	60	90	120
K	0.5	1.4	0.7	—	—	—
		0.5	—	—	—	—
		0.7	—	—	—	—
Dihydro F	0.25	0.5	0.415	—	—	—
		0.5	0.35	—	—	—
		0.5	0.25	—	—	—
		0.7	0.35	—	—	—
		0.7	0.35	—	—	—
G	0.15	0.6	0.42	0.2	—	—
		1.25	0.75	0.15	—	—
X	0.05	0.67	0.67	0.325	0.1	0.05
		1.5	1.0	0.5	0.25	0.14

TABLE II.
Time Relationships of the Urinary Excretion of Penicillins X, G, Dihydro F and K During a 6-Hour Period After Intramuscular Injection of 300,000 Units.

Penicillin	Subject	% of total 6-hr excretion recovered in each 2-hr period 0-2 hr	2-4 hr	4-6 hr
X	Re	86	12	2
	Ha	80.5	18	1.5
	Avg	83.2	15	1.75
G	Sh	96	2.8	1.2
	To	91.6	6.6	1.8
	Eb	84.7	12.1	3.2
	Ku	86	10.5	3.5
	Mc	85.5	12	2.5
	Avg	88.9	8.8	2.4
Dihydro F	Re	89.3	9.9	0.8
	Ba	99	0.4	0.6
	Ti	91	7.1	1.9
	Gu	80	18.0	2.0
	Fe	88	9.6	2.4
	Avg	89.4	9.0	1.5
K	Be	70	17	13
	Fe	32.7	52	15.3
	Ka	67.5	27.5	5
	Re	77	22	1
	Ro	76	16.6	7.4
	Avg	64.6	27	8.3

distinctly different from the other 3 penicillins, in that relatively high percentages of the total excretion occur after the first 2 hours. Moreover, the average renal clearance of penicillin K in the 5 subjects was less than half that of penicillins G and X.

Discussion. From the data presented it appears that consideration of the antagonistic action of serum, which presumably occurs as a result of protein-binding, materially alters the previous interpretations of the serum levels attained after intramuscular administration of the crystalline penicillins studied. The serum concentrations of penicillins X, G, dihydro F and K attained after intramuscular injection were remarkably uniform during the period when all 4 were measurable by a method which allows constant conditions of

binding by serum protein. The 2 chief features previously reported for the individual penicillins, *e.g.*, higher and more prolonged serum concentrations afforded by penicillin X, and lower and poorly sustained concentrations by penicillin K, were not observed.

Of particular interest was the observation that in each of the 5 subjects who received 300,000 units of penicillin K, the serum concentrations at 60 minutes were precisely the same as had been present 15 minutes after administration. The explanation of this is not apparent. In view of other observations on penicillin K, and its similarity to the other penicillins, it is conceivable but unlikely that the sustained serum concentration during the first hour is the result of delayed absorption. The findings may reflect the fact observed by Richardson and his co-workers, that penicillin K is distributed in the body differently than the other penicillins. Alteration in renal excretion caused by protein-binding might also contribute to this phenomenon of a constant concentration during the first hour after administration.

Previous observations which originally led to the assumption that penicillin K is rapidly destroyed *in vivo* are worthy of critical analysis. If true, the occurrence of destruction would almost necessarily militate against the use of penicillin K therapeutically. In contrast, if penicillin K is largely bound by plasma protein *in vivo,* even though the drug is inactive when bound, it would eventually become free and potentially active as a result of the reversibility of the process of binding. It is obviously of importance to know more exactly the pharmacologic behavior of penicillin K, for a penicillin which is *not* rapidly eliminated by the kidney unquestionably would have distinct advantages, provided it were not more susceptible to destruction *in vivo.* The observations reported here and those of Richardson and his associates[9] strongly suggest that whatever mechanisms are in operation to bring about the fall in serum concentration of penicillin K after intramuscular injection, they do not remove penicillin K more rapidly than does the process of urinary excretion in the case of the other penicillins.

One might anticipate from these findings that the actual mode of administration of penicillin K could profoundly affect its comparative therapeutic effectiveness. It is of interest in this respect to contrast the results obtained by Hobby and her associates[3] and by Eagle[4] in the treatment of experimental hemolytic streptococcal infections in mice. The same infecting organism was used by both investigators. Eagle treated the animals with 10 equal doses of penicillin in saline solution administered at intervals of 3 hours. In contrast Hobby employed only 3 injections of penicillin suspended in peanut oil, and gave the injections as follows: (1) 40% of total dose immediately after infection, (2) 40% of total dose 6 hours later, and (3) 20% of the total dsoe 16 hours after the second injection. When penicillin X and penicillin G were compared in this way both investigators found virtually the same unitage ratios of X to G, *e.g.,* 480:100 (Hobby) and 500:100 (Eagle). Comparison of G with K, however, showed a marked difference between the 2 methods of study. On a unitage basis, Eagle found the G:K ratio to be 100:7, whereas Hobby found it to be 100:60. Several factors may contribute to the difference in relative effectiveness of penicillin K observed by the 2 investigators. It would appear, however, that the chief differences between the 2 studies were the size of the individual doses, and the interval between injections. Penicillin K appeared to be significantly more effective as compared with penicillin G when given in larger individual doses at longer intervals. This is in accord with the facts that penicillin K is bound to a high degree by albumin and has a low renal clearance.

The data presented on penicillin dihydro F are the first studies reported in humans. Penicillin dihydro F in general exhibited a higher degree of binding than penicillin G, and its lowest measurable level in serum was intermediate between penicillins G and K. The calculated rate of fall in serum concentration of penicillin dihydro F is slightly greater than that of G, but the difference is

probably not of significance. In this respect it should be mentioned that the crystalline sample of penicillin dihydro F used in these studies may have contained 10-20% of "K-type" penicillins. Preliminary experiments on certain mixtures in serum of 2 penicillins which are bound to greatly different degrees suggest that the observed activity is solely that of the penicillin which is bound to the lesser degree. For example, pooled serum containing mixtures of penicillins G and K over a wide range of ratios of concentration, when assayed by the technic employed in these studies has consistently shown an apparent activity equal to that of the penicillin G present. Thus the values obtained for serum concentration after administration of penicillin dihydro F may actually represent what would be obtained after approximately 80% of the dose were given in the form of pure material. This probably would not differ significantly from the results which have been presented.

Summary. The absorption and excretion of penicillins X, G, dihydro F and K in humans have been studied in an effort to evaluate the influence of the marked differences in the degrees of binding of these substances by serum protein.

The duration of the serum concentrations afforded by all 4 penicillins after intramuscular injection of 300,000 units to humans were uniform within the limits of the method of assay. During the period when all of the penicillins were present in measurable concentrations there was no difference among the 4 except for the lower peak of penicillin K 15 minutes after injection. Evidence was presented that previous findings of higher and more prolonged serum concentrations of penicillin X, and lower, rapidly disappearing concentrations of penicillin K were artifacts created by differences in the antagonistic action of the serum on penicillin during the actual bioassay procedure. Thus differences in therapeutic effectiveness of the individual penicillins cannot be explained on the basis of differences in height and duration of serum concentrations after equal doses.

Although the total excretion of penicillin K was low, the rate of excretion and the renal clearance suggest that the mechanism of removal of penicillin K by the kidney differs from that observed with the other penicillins.

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Antidotal Properties of Crude *Penicillium notatum* Filtrate.

J. SMOLENS, D. S. MCALEER, AND C. S. MCLAREN. (Introduced by M. Landy.)

From the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

Recent work of Ramon, *et al.* shows that there is present in filtrates of crude *Penicillium notatum* an agent which detoxifies various toxins.[1-3] This property they have called "antidotal." According to these workers this action is enzymatic and they conclude that preparation of highly purified penicillin results in a loss of this factor which, from a therapeutic point of view, may be unwise. No *in vivo* therapy data were given. The following is a summary of *in vitro* and *in vivo* experiments determining the antidotal efficacy of crude penicillium broth.

Materials. Toxin—crude tetanus which assayed about 100,000 LD/ml.

P. notatum filtrate—a crude commercial filtrate from which the penicillin had been extracted.

Mice—white, inbred strain, either sex, 18-21 g.

Broth—1% peptone.

Experimental. To make the experiment

1 Ramon, G., Richou, R., and Ramon, P., *C. R. acad. sci.*, 1946, **222**, 621.

2 Ramon, G., Richou, R., and Ramon, P., *La Presse Medicale*, 1946, Oct. 2.

3 Ramon, G., Richou, R., and Ramon, P., *Rev. Immunol.*, 1944-45, **9**, 161.