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Studies on the Stability of Drugs in Biological Media. (2).[1] Relationship between the Stability in Culture Media and Anti-bacterial Activity of Some Isoniazid Derivatives[2]

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The relationships between stability in culture media and *in vitro* anti-bacterial activity against *Bacillus Calmette-Guérin* (*BCG*) were investigated with furyl methyl ketone isonicotinoylhydrazone (FKI), glucose isonicotinoylhydrazone (G-INAH), and sodium pyruvate isonicotinoylhydrazone (P-INAH). FKI was found to be essentially equal to isoniazid in its activity under the equimolar basis. Thus its stability which was dominantly affected by several amino acids resulted to have no influence on the evaluation of the activity. On the other hand, a significant relationship was found among G-INAH and P-INAH, which can not inhibit growth of *BCG* until they release sufficiently large amount of isoniazid by hydrolysis. P-INAH was found to be hydrolyzed with such an extraordinary rapidity as 6 and 23 times greater than G-INAH in Kirchner and Long medium, respectively. This instability inversely related to the enhancement of its activity which consequently resulted in the same one as isoniazid. While G-INAH was observed to be hydrolyzed in Kirchner medium about 4 times as rapidly as in Long medium and it showed to have greater activity in the former than in the latter. The release rate of isoniazid might be inversely proportional to the stability and directly to the activity of these drugs. Another *in vitro* experiment with drug exposure times suggested that the bactericidal effect of drugs was probably displayed in the relatively early stage of test incubation period.

Anti-bacterial agents which are shown to be unstable in aqueous solution or nutrient broth include benzylpenicillin,[4] methicillin,[5] cephalothin,[6] and some anti-tuberculous agents like glucuronolactone isonicotinoylhydrazone[7] or furyl methyl ketone isonicotinoylhydrazone.[1] Wick studied consequence of the drug instability in conventional anti-bacterial sensitivity test with cephalothin,[6] while Colwell used glucuronolactone isonicotinoylhydrazone for the same purpose.[7] Using some anti-tuberculous agents, Kanai has suggested that stability of drug must be taken into consideration in the case of *in vitro* activity test of essentially bacteriostatic drugs or even in the bacteriostatic concentrations of bactericidal ones.[8] Their studies, however, are largely of qualitative nature.

The primary purpose of this work is to evaluate quantitatively the relationship between the stability in culture media of various anti-tuberculous agents and their activity *per se* against *Bacillus Calmette-Guérin* and to contribute to a rational approach to the evaluation of *in vitro* activity of investigational drugs. For this purpose, three isoniazid hydrazones including furyl methyl ketone isonicotinoylhydrazone (FKI) were chosen as model compounds.

1) Part (1): K. Kakemi, H. Sezaki, N. Takasugi, and K. Iwamoto, *Chem. Pharm. Bull.* (Tokyo), **16**, 1481 (1968).
2) Presented at the 87th Annual Meeting of Pharmaceutical Society of Japan, Kyoto, April 1967.
3) Location: *Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto.*
4) F.P. Doyle, J.H.C. Nayler, H. Smith, and E.R. Stove, Nature, **191**, 1091 (1961).
5) F.P. Doyle, A.A.W. Long, J.H.C. Nayler, and E.R. Stove, *Nature.*, **192**, 1183 (1961).
6) W.E. Wick, *J. Bacteriol.*, **87**, 1162 (1964).
7) C.A. Colwell and A.R. Hess, *Am. Rev. Tuberc. Pulmonary Diseases*, **73**, 892 (1956).
8) K. Kanai, S. Okamoto, and T. Murohashi, *Kekkaku*, **38**, 512 (1966).

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Experimental

Materials——FKI (Daiichi Seiyaku Co., Ltd.), isoniazid (Takeda Chemical Industries Ltd.) (INAH), and sodium pyruvate isonicotinoylhydrazone (Tanabe Seiyaku Co., Ltd.) (P-INAH) were used as received. Glucose isonicotinoylhydrazone (G-INAH) was prepared by the method previously reported.[9] All other chemicals used were of reagent grade.

Procedure for Kinetic Studies of G-INAH and P-INAH in Culture Media——Kirchner and Long media were used after adjusting pH to 7.0 and 6.0 respectively by sodium hydroxide solution. A series of broth cultures containing drugs in an appropriate concentration were prepared in volumetric flasks and allowed to incubate in a constant temperature bath kept at 37°. Aliquots were withdrawn periodically and analyzed with respect to isoniazid. Isoniazid concentrations were determined spectrophotometrically by the β-naphthoquinone-4-sodium sulfonate (NQ) method[10] using a Shimadzu model QV-50 spectrophotometer. To avoid interference by asparagine contained in broth, optical density was measured exactly 1 minute after the addition of NQ reagent.

Drug-Sensitivity Tests of Tubercle Bacilli——*Bacillus Calmette Guérin* (*BCG*), a mutant of *Mycobacterium tuberculosis*, was used as a test organism. Weighed organisms of a 14-day-old culture in Ogawa medium were ground thoroughly to make a suspension of 1 mg per ml. Minimal inhibitory concentrations (MIC) were determined by the conventional tube dilution method with Kirchner and Long medium. Each tube which contained 4 ml of medium was inoculated with 0.1 ml of supernatant of the organisms suspension and added with 1 ml of drug solution which was prepared by a serial dilution. MIC's were determined after 2 or 3 weeks incubation at 37°. All the procedures were carried out aseptically.

Effect of Varying Drug-Exposure Times on a Growing Culture——Barclay's method[11] was employed with some modifications and the procedure is given in Chart 2. Bacterial growth was analyzed daily by measuring the turbidity using a Hitachi Perkin-Elmer model 139 spectrophotometer.

Results and Discussion

Stability of G-INAH and P-INAH in Culture Media

As shown in Fig. 1, a linear relationship between time and logarithm of residual drug concentration in media was obtained in both cases, indicating that the degradation of both drugs obeys first-order kinetics as that of FKI.[1]

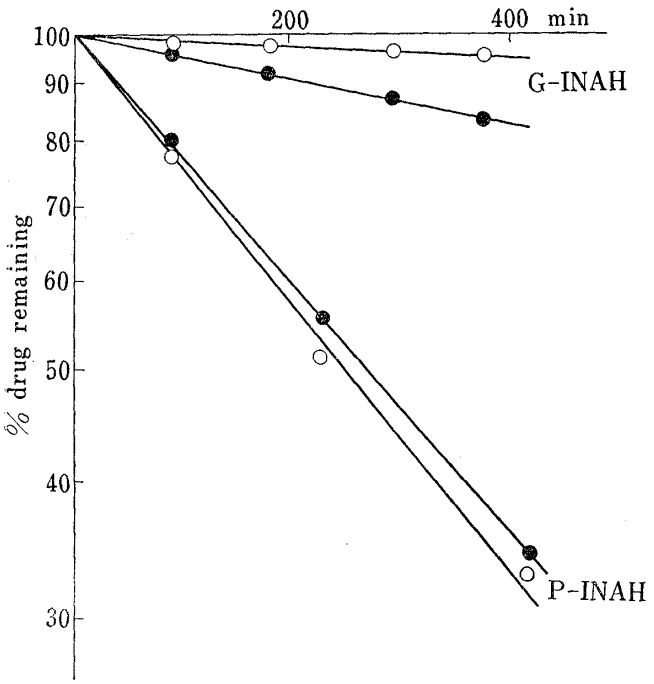

Fig. 1. Stability of G-INAH and P-INAH in Kirchner (●) and Long (○) Media at 37°

The proposed route of hydrolysis is given in Chart 1. Table I summarizes rate constants and the half-lives of the degradation of these drugs, including FKI for the comparison.[1] As compared with G-INAH or FKI, P-INAH was found to be hydrolyzed with extraordinary rapidity in both media.

It would be expected that considerably larger amounts of free isoniazid are released from P-INAH by hydrolysis than G-INAH or FKI, long before the growth of organisms. In Kirchner medium, G-INAH was hydrolyzed about 4 times as rapidly as in Long medium. It was also

9) H. Zinner and W. Bock, *Chem. Ber.*, **89**, 1124 (1957).
10) H. Fujiwara, *Yakugaku Zasshi*, **78**, 1034 (1958).
11) W.R. Barclay and E. Winberg, *Am. Rev. Respirat. Diseases*, **90**, 749 (1964).

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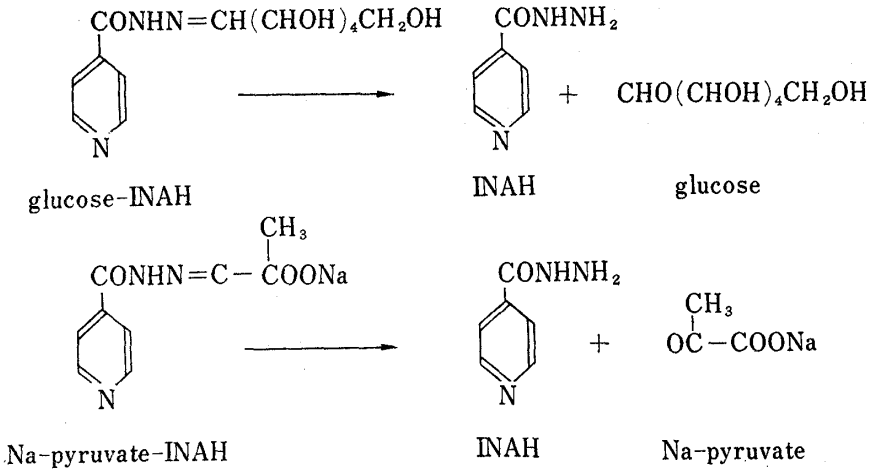

Chart 1. Hydrolysis of Glucose-INAH and Na-Pyruvate-INAH

Table I. Stability of Isonicotinoylhydrazones in Culture Media

Compounds	Degradation rate constant[a] and half-life[b] In Kirchnner medium	In Long medium
G-INAH	4.56 (1.06)	1.23 (3.91)
P-INAH	25.3 (0.19)	28.6 (0.17)
FKI[c]	4.01 (1.20)	8.75 (0.55)

a) Given in the first half of the column ($10^{-4}min^{-1}$).
b) Given in the parenthesis (day).
c) Taken from reference 1).

found that G-INAH releases larger amount of isoniazid in Kirchner medium than in Long medium.

Drug-Sensitivity Tests

The *in vitro* activity data of FKI has been reported to vary ranging from almost equal to hundred times as potent as the parent isoniazid[12] and the question appears to be whether the salutary effects of the drug are merely due to the release of isoniazid contained in its molecule or it acts as a separate drug. Previous work from this laboratory has shown that FKI is degraded four to ten times more rapidly in nutrient media than in buffer solutions and its instability is greatly affected by medium components which exhibit dominant effect in catalysis of FKI hydrolysis.[1] Influence of stability of FKI on its *in vitro* activity test was investigated

Table II. Comparison of Minimal Inhibitory Concentrations of INAH and its Derivatives against BCG

Compounds	MIC (10^{-7} M)[a] In Kirchner medium[b]	In Long medium[c]
INAH	8.0	8.0
FKI	8.0	8.0
G-INAH	40.0	120.0
P-INAH	8.0	8.0

a) Tested in more than triplet.
b) and *c*) Determined after incubation for 14 and 21 days, respectively.

12) *a*) K. Miyatake, S. Ichimura, S. Nagasaki, and K. Hoji, *Yakugaku Zasshi*, **75**, 1066 (1955); *b*) E. Genazzani and A. Paoletti, *Minerva Medica*, **49**, 4951 (1958); *c*) S. Oka, S. Kudo, and E. Kitajima *Nihon Kyobu Rinsho*, **21**, 450 (1962).

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using 10% bovine serum added Kirchner medium. This medium was prepared so as to contain one amino acid in turn as nitrogen source. However the attempt failed under the conditionsemployed, since the growth of BCG could not be detected at all in the medium containing other amino acids than asparagine. This may be explained by the finding of Yamamura who has shown strain-specificity for utilization of nitrogen source in the study using four strains of *Mycobacterium tuberculosis* and suggested that asparagine was most effectively assimilated by the organisms as indispensable nitrogen source.[13]

The activity data of four drugs are shown in Table II. MIC was determined after incubation for 14 and 21 days culture in Kirchner and Long media, respectively .It is evident from the result that FKI was demonstrated to have the same activity under the equimolar basis (8×10^{-7} M) as isoniazid and there was no difference in their MIC's between the cultures in both media. P-INAH was also shown to have the same activity (8×10^{-7} M) as isoniazid. The MIC's of G-INAH were, however, greater than that of iso niazid and it apparently showed greater activity in Kirchner medium (4×10^{-6} M) than in Long medium Table II (1.2×10^{-5} M).

These data could lead the following conclusions. FKI *per se* is substantially equal to isoniazid in its activity against BCG, while P-INAH and G-INAH, which are inert in intact form mainly because of their poor lipid solubility, did not inhibit growth until they release sufficient isoniazid. Apparently P-INAH resulted in the same activity as isoniazid owing to a sufficiently large amount of isoniazid released by rapid hydrolysis, while G-INAH exhibited less activity than isoniazid because of a relatively small amount of isoniazid released. Colwell has indicated in the same line that the origin of the action of glucuronolactone isonicotinoylhydrazone was free isoniazid released in culture medium.[7]

Drug-Exposure Time Effect

It is interesting to note that although a fairly large portion of G-INAH was in the form of free isoniazid in both media after usual long term incubation period such as 14 to 21 days, the activity data were definitely dependent on the initial rate of hydrolysis. Studies by Barclay with C^{14}-labelled isoniazid have revealed a very rapid uptake of the drug by susceptible bacteria in both a growing and a dormant state.[14] In his recent work he described more precisely the relationship between the duration of isoniazid exposure and the effectiveness of the drug.[15]

With the above in mind, it became desirable to investigate the apparent activity of these compounds as a function of duration of exposure. Test procedures are given in Chart 2.

add 10 ml of drug solution (5×10^{-4} M) to 30 ml of 4-day-old culture of *BCG* in 10% bovine serum containing Kirchner medium and expose for 2, 4, and 7 hours under incubation at 37°

↓

centrifuge the exposed tube at 3000 rpm for 15 min and remove the drug solution by decantation

↓

wash the cells twice with 25 ml of fresh Kirchner medium and add 50 ml of the same medium to the drug-free cells

↓

incubate the cells at 37° and measure the bacterial growth daily by turbidity reading at 630 mμ

Chart 2. Test Method of BCG Exposure to Drug

13) Y. Yamamura, *Kekkaku*, 27, 450 (1962).
14) *a*) W.R. Barclay, R.H. Ebert, and D. Koch-Wester, *Am. Rev. Tuberc. Pulmonary Diseases*, **67**, 490 (1953); *b*) W.R. Barclay, D. Koch-Wester, and R.H. Ebert, *ibid.*, **70**, 784 (1954).
15) W.R. Barclay and E. Winberg, *Am. Rev. Respirat. Diseases*, **90**, 749 (1964).

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In the stage of pre-incubation before the exposure tests, serum was added to the medium as a nutrilite for the well-growth of *BCG*.[16] Drug concentration (1.25×10^{-4} M) in the exposure was sufficiently high above the MIC's obtained before. The organisms were exposed early in the logarithmic phase of their growth. Manifestations of bactericidal action of isoniazid in relation to the culture age of cells in liquid medium have been studied by Hobby.[17] However in this study tested by using 4- and 7-day-old cultures of *BCG*, the activity of four drugs was not influenced by the culture age of the cells. Furthermore the stability of the drugs was not affected at all in the presence of the cells.

The effect of exposure times on the growth of *BCG* is shown in Fig. 2, where closed circle (0 hour) indicates the growth of *BCG* without exposure. The growth curve of *BCG* exposed to FKI was similar to that exposed to isoniazid, while those exposed to G-INAH and P-INAH were somewhat steeper in comparison with the former. According to Barclay,[14] the population of unaffected organisms $(1-p)$ could be calculated from the following equation,

$$(1-p)=Nn—Nm/Qe^{kn}—Qe^{km}$$

where Nm an Nn are the nephelometric readings of exposed organisms at time m and n in day respectively, and Qe^{km} and Qe^{kn} express those of untreated control culture.

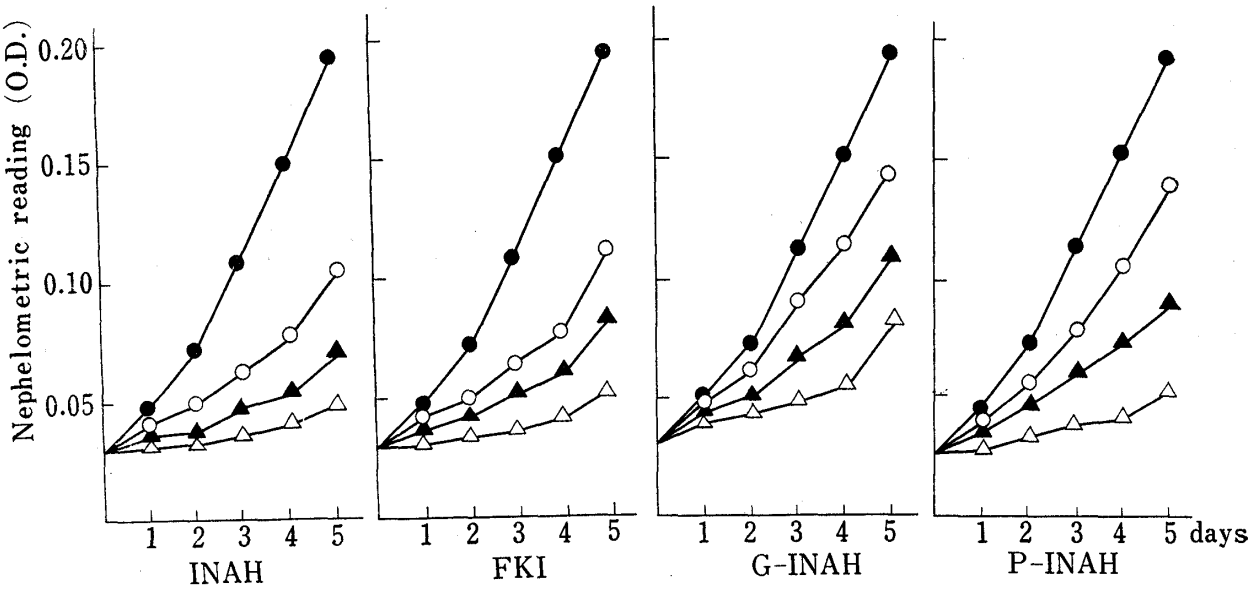

Fig. 2. Effect of Varying Exposure Times on Growth Culture of *BCG*, Nephelometric Reading Plotted against Time (day) after Exposure Test for 0 (●), 2 (○), 4 (▲), and 7 (△) hours

The percentages of organisms surviving after various time exposures to drugs were calculated by the above equation and are plotted against exposure times in Fig. 3. Time m and n were selected as 2 and 3 days, when the unexposed control culture began to grow logarithmically. In both cases of isoniazid and FKI, a straight line was obtained. These lines express the probability of mycobacterial death as a function of duration of exposure. Extrapolating the line to 0-time gives a theoretical estimate of the percentage of organisms killed by instantaneous exposure. This was about 50% of the organisms in the case of isoniazid and FKI, but almost none in G-INAH and P-INAH. Since almost all of the organisms are supposed to be killed by the exposure to these four drugs within 8 hours under the conditions employed, the sensitivity data of G-INAH may be interpreted in such a way that the bactericidal activity would be exerted in an early stage culture incubation.

16) M. Nakamura, *The Kurume Medical J.*, **2**, 51 (1955).
17) G.L. Hobby and T.F. Lenert, *Am. Rev. Tuberc.*, **76**, 1031 (1957).

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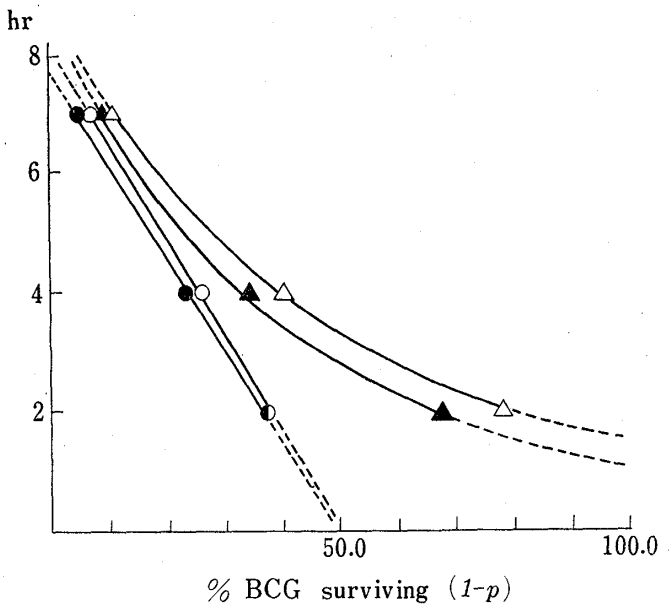

Fig. 3. Relationship between Exposure Time and Percentage of *BCG* surviving after Exposure to INAH (●), FKI (○), G-INAH (△), and P-INAH (▲)

As mentioned by Barclay,[15] a culture of *BCG* also appears to have a population susceptible and another population nonsusceptible to drugs, and the former is to be killed by the exposure in an early stage of incubation.

These observations might give a suggestion with a great significance that reading the end point after the standard long incubation period might give a misleading impression of activity and that the rate of degradation of drugs in relatively early stage of incubation must be taken into the consideration for the *in vitro* evaluation of the potential activities of antibacterial agents.

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