gen, glycerol, or 2,3-butylene glycol could be detected, and only traces of acetyl methyl carbinol or diacetyl could be shown. A fair carbon and hydrogen balance[5] was obtained for the fermentation of glucose in a medium initially containing 326 mg glucose, 45 mg Difco yeast extract, 3 mg proline, 3 mg phenylalanine, 1.5 mg cystine, 1 mg isoleucine, 1 mg valine, 45 mg NH_4Cl, 45 mg $MgSO_4 \cdot 7\ H_2O$, 2 mg $CaCl_2$, and 1 mg $FeCl_3$ per 100 cc of 0.05 M Sorensen phosphate buffer at pH 7.5. During the fermentation the medium was neutralized at intervals with NaOH, until all of the sugar had disappeared. The results are presented in Table I.

Although the carbon recovery appears to be satisfactory, the ratio of two-carbon to one-carbon derivatives (see 5) is extraordinarily high for an ordinary fermentation, being approximately 3.2. This might indicate that a portion of the one-carbon derivatives remains unaccounted for (as unidentified products in the medium or in the bacterial) or, perhaps that part of the 2-carbon compounds may arise, not through the splitting of a 3-carbon degradation product of sugar, but by some other method such as the reduction of carbon dioxide.

[5] Doudoroff, M., *J. Bact.*, 1942, **44**, 461.

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Resistance of Small Colony Variants (G-Forms) of a Staphylococcus Towards the Bacteriostatic Activity of Penicillin.

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So far, two types of penicillin-resistant staphylococci have been described:

(1) Naturally resistant strains, as reported by Fleming,[1] and by Hobby, Meyer and Chaffee.[2] We, too, found 5 resistant strains of *Staphylococcus aureus* in a group of 12.

(2) Strains rendered drug-resistant by prolonged contact of the organisms with increasing concentrations of penicillin (Abraham and co-workers,[3] Smith and Hay,[4] Rammelkamp and Maxon.[5]) These strains may exhibit slight morphological changes, such as larger size of the coccus[4] or a reduced velocity of growth and enzyme activity.[3]

A third type of penicillin-resistant staphylococci, which has not been described before, is represented by variants occurring when a normal strain of *Staphylococcus albus* was exposed to penicillin. Such strains were studied in our laboratory during recent months. They show the profound cultural changes known as small colony variants (Hoffstadt and Youmans,[6] Swingle,[7] Youmans,[8] Youmans and Delves.[9])

Material and Methods. The penicillin used was produced by growing a strain of *Penicillium notatum* No. 1209* in a modified

[1] Fleming, I., *Lancet*, 1942, **1**, 732.

[2] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

[3] Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, A. M., *Lancet*, 1941, **2**, 177.

[4] Smith, L. D., and Hay, T., *J. Franklin Instit.*, 1942, **233**, 598.

[5] Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

[6] Hoffstadt, R. E., and Youmans, G. P., *J. Infect. Dis.*, 1932, **51**, 216.

[7] Swingle, E. L., *J. Bact.*, 1935, **29**, 467.

[8] Youmans, G. P., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 94.

[9] Youmans, G. P., and Delves, E., *J. Bact.*, 1942, **44**, 127.

* We are indebted to Dr. Robert D. Coghill, Bureau of Agricultural Chemistry and Engineering, Northern Regional Research Laboratory, Peoria, Ill., for the strain of *Penicillium notatum* and of *Staphylococcus albus* No. 314.

Czapek-Dox medium.[3] Initial experiments were conducted with Seitz filtrates of the crude culture fluids, containing 4-8 Oxford units per ml, while the later work was carried out with concentrates of barium penicillate containing 500 Oxford units per ml.† The values given for penicillin are expressed in Oxford units as defined by Florey and Jennings.[10]

All experiments were conducted with a strain of *Staphylococcus albus* No. 314* as the test bacterium. The media for culturing the Staphylococcus and for all the tests carried out in these studies were papain digest broth and agar prepared according to the method of Asheshov.[11]

The ring test (Abraham and co-workers[3]) and the dilution method in liquid media were used for measuring the sensitivity of our strains. The dilution or titration method was carried out in test tubes containing serial dilutions of penicillin in a volume of 5 ml. The tubes were seeded with 0.1 ml of a 20-hour broth culture and the results read after 18 to 20 hours incubation. *In vitro* tests with methyl violet were carried out similarly but the volume was only 2 ml.

Experimental. In a ring test with *Staphylococcus albus* No. 314 a crude culture fluid of *Penicillium notatum* produced an area of inhibition of 25.5 mm diameter. This area was covered almost completely with a thin film of minute translucent colonies of staphylococci. This growth was obviously identical with the ghost-like "halos of partial inhibition" mentioned briefly but not further investigated by Abraham and co-workers.[3] By subculturing some of the minute colonies a fair growth of these very small colonies was obtained. They exhibited the morphological and cultural characteristics of small colony variants described by former investigators.[6,7,8,9] Two days later a few colonies of normal appearance were found in subcultures but the small colonies could be isolated again and propagated on liquid and solid media. This strain of small colony variants (No. 314 G) was then split up in 3 cultures which were transferred daily on agar plates for 20 days. One culture was kept on plain papain digest agar (No. 314 G a), the other on the same agar base containing 10% of a Seitz filtrate of culture fluid of *Penicillium notatum* (No. 314 G b), and the third culture (No. 314 G c) was kept on agar containing 1% of the same culture fluid. The strains grew abundantly on these media, without any reversion to the normal growth. If kept on agar plates and slants with only one weekly transfer to fresh medium, the 3 strains were stable. During 4 months no reversion was observed. However, if daily transfers were made on rich media, such as beef heart infusion and yeast extract broth and agar, all 3 G-forms reverted to the normal type of growth within one week.‡

The most interesting property of the small colony variants was their resistance to penicillin. In frequent ring tests carried out in triplicate the parent strain showed consistently normal sensitivity with an area of inhibition of 19 to 25 mm. Small colony variants were not inhibited at all, while reverted strains regained the sensitivity of the parent strain. There was no difference in penicillin resistance between the strains No. 314 G a, No. 314 G b, and No. 314 G c, indicating that the single initial exposure to penicillin had sufficed to produce a stable penicillin-resistant G-form.

In order to find out whether the resistance was specific, *i.e.*, due to the primary exposure to the bacteriostatic agent, the modified strains produced by penicillin and variants obtained by exposure to $BaCl_2$[8,9] were compared with respect to their sensitivity to penicillin concentrate and to methyl violet.

By growing Staphylococcus No. 314 in papain digest broth containing 2%, 1%, 0.5% $BaCl_2$, abundant formation of G-forms occurred after 48 hours incubation. Three strains, marked No. 314 G B 2, No. 314 G B 1, and No. 314 G B 05, were isolated from the 3 $BaCl_2$ media. They yielded typical small colony variants; Strain No. 314 G B 05,

† Prepared by Drs. Goldberg, Oppenheim-Errera (deceased), and Scott of the Roche Laboratories.

[10] Florey, H. W., and Jennings, M. A., *Brit. J. Exp. Path.*, 1942, **23**, 120.

[11] Asheshov, I. N., *Canad. Pub. Health J.*, 1941, **32**, 468.

‡ Observations made by B. Tabenkin of the Roche Laboratories, who also supplied us with the crude culture fluids of *P. notatum*. Grateful acknowledgment is made of his cooperation.

TABLE I.
Sensitivity of Staphylococcus No. 314 and Its Small Colony Variants to Penicillin and Methyl Violet.

Strain	Variants produced by exposure to	Inhibition by penicillin: Dilution method Oxford units/ml	Inhibition by penicillin: Gutter plate, mm*	Inhibition by methyl violet
No. 314	—	0.002	19-19.5	1-1280 × 1000 -2560 ,,
,, G a	Penicillin	>1	0	1-320 ,,
,, G b	,,	>1	0	1-320 ,,
,, G c	,,	>1	7.5	1-320 ,,
,, G B 2	$BaCl_2$	—†	—	1-320 ,,
,, G B 1	,,	0.125	5	1-320 ,,
,, G B 05	,,	0.5	0	1-320 ,,
,, G B 2 reverted	,,	—	20	—

* Distance from the edge of the gutter to the zone of full growth.
† The strain was lost at the time of these experiments.

derived from the medium with 0.5% $BaCl_2$, was the only one which showed less translucent colonies with a slightly whitish surface. The strains were fairly stable if overnight cultures on agar slants were kept at room temperature and not transferred more frequently than once a month.

In Table I the results of the *in vitro* experiments with the original strain and its different variants are tabulated. The penicillin used in these experiments was a concentrate of barium penicillate containing 500 Oxford units per ml. The agar strip of the gutter plate (Fleming[1]) was prepared with an aqueous solution of barium penicillate and contained 30 Oxford units per ml.

The table demonstrates the greatly increased resistance of the small colony variants in comparison with the normal sensitivity of the original strain and of one of the reverted cultures. Moreover, all small colony variants show a markedly reduced sensitivity towards the bacteriostatic activity of methyl violet.

Discussion. The observations presented in the present paper indicate that the small colony variants of a strain of *Staphylococcus albus* are resistant to penicillin. Although strains obtained by previous exposure to penicillin appear slightly more resistant than the strains produced by exposure to $BaCl_2$, these differences can hardly be considered significant. This resistance to penicillin is not a specific drug-fastness, but seems to be part of the profound modification undergone by these dissociated strains. The reduced sensitivity of the small colony variants to a completely different chemical agent, such as methyl violet, seems to substantiate this view and indicates that these variants acquired a non-specific resistance towards specific antibacterial agents. This is a phenomenon similar to the resistance of small colony variants of staphylococci to staphylococcus bacteriophage as described by Hoffstadt and Almaden.[12] It is interesting to note that Philipps and Barnes[13] encountered 6 different forms of variants when they produced resistance of *Staphylococcus aureus* towards gramicidin. By eliminating the variants, they obtained gramicidin-resistant strains with otherwise normal cultural appearance, enzyme activity, and unchanged sensitivity to crystal violet. Strains like these probably belong in the same group as the tyrothricin-resistant staphylococci described by Rammelkamp[14] and the penicillin-resistant strains of Abraham and co-workers[3] and Rammelkamp and Maxon[5], and may represent examples of genuine and specific drug-fastness. The resistance exhibited by our strains of small colony variants is not specific.

Summary. 1. Small colony variants (G-forms) of a strain of *Staphylococcus albus* were obtained on agar by the action of penicillin. 2. These variants showed a high

[12] Hoffstadt, R. E., and Almaden, P., *J. Infect. Dis.*, 1934, **54**, 253.

[13] Philipps, R. L., and Barnes, L. H., *J. Franklin Instit.*, 1942, **233**, 396.

[14] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 346.

resistance towards the bacteriostatic activity of penicillin. 3. Small colony variants of the same strain of *Staphylococcus* produced by the exposure to $BaCl_2$ possessed likewise a considerable resistance to penicillin. 4. All these strains of small colony variants regardless of their origin acquired also a certain degree of resistance towards methyl violet. 5. The resistance of these strains of small colony variants towards penicillin cannot be considered to be a specific drug-resistance.

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Effect of Neosynephrin* on Gaseous Exchange of the Brain.†

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The effects of neosynephrin on the cerebral arterio-venous oxygen difference were studied on large dogs, narcotized with Dial,‡ 0.5 cc per kilo. The cranium was trephined above the superior longitudinal sinus, the trachea was exposed and cannulated and both femoral arteries were also exposed. The first pair of femoral arterial and cerebral venous blood samples was collected while the animals were breathing air and the second after they had come to equilibrium while respiring a gas mixture containing approximately 11% oxygen. Neosynephrin was then administered and when blood pressure had risen significantly the third pair of blood samples was collected. Blood flow of the cerebral longitudinal sinus was determined by Daly's modification of the thermostromuhr.[1] Blood pressure was recorded by means of a cannula inserted into a femoral artery. Blood samples were analyzed for oxygen and carbon dioxide contents and oxygen capacity,[2] for glucose,[3] and for lactic acid.[4] The oxygen tension of the blood was estimated by using the nomogram of Bock *et al.* for humans[5] which may also be applied to dogs.[6] In addition, the effects of neosynephrin on the cerebral arterio-venous oxygen difference were determined on dogs breathing room air. In most experiments 2 cc of 1% neosynephrin solution were administered intramuscularly, portions of the 2 cc being injected at various sites. In a few experiments, however, 1-10,000 neosynephrin solution was injected intravenously and in these instances the amount of neosynephrin injected was much smaller than with intramuscular injection.

In Table I are presented the results of 2 out of 4 experiments on animals breathing reduced concentrations of oxygen. In all 4 experiments the blood became more alkaline as a result of the hyperpnea produced by breathing 11% oxygen and then less alkaline after injections of neosynephrin. Neosynephrin increased the concentrations of lactic acid and glucose in the blood. Blood pressure was

* We are grateful to Dr. Richard M. Johnson of the Frederick Stearns & Company for his generous supplies of Neosynephrin Hydrochloride.

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‡ We are grateful to Dr. E. Oppenheimer of the Ciba Pharmaceutical Products for his generous supplies of Dial Urethane Solution.

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3 Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 63.

4 Friedemann, T. E., Cotonio, M., and Shaffer, P. A., *J. Biol. Chem.*, 1927, **73**, 335.

5 Bock, A. V., Dill, D. B., Hurxthal, L. M., Lawrence, J. S., Coolidge, T. C., Dailey, M. E., and Henderson, L. J., *J. Biol. Chem.*, 1927, **73**, 749.

6 Dill, D. B., Edwards, H. T., Florkin, M., and Campbell, R. W., *J. Biol. Chem.*, 1932, **95**, 143.