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TRACER DYE GREEN S (FOOD GREEN NO. 4) IN PENICILLIN PREPARATIONS FOR INTRA- MAMMARY APPLICATION, II.¹⁾

By

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Rasmussen & Simesen (1960) added 50 mg. Green S (Edicol Supra Green B S — Food Green No. 4, New C. I. 44090) per 100,000 I.U. of penicillin to six different preparations for intramammary infusion and found a direct relationship between the concentrations of penicillin and tracer dye in the milk produced by the treated cows, even though the excretion periods for the various preparations ranged from 60 to 460 hours. In these experiments it was further shown that preparations containing aluminium monostearate might show a very prolonged excretion — cf. also *Simesen* (1959).

Since the maintenance of low penicillin concentrations in the milk for up to 3 weeks after the infusion must be considered undesirable, from a food-hygienic as well as from a therapeutic aspect (*English* 1959; *Simesen* 1959; *Høgh* 1960), attention was directed towards the development of preparations with a constant, short excretion time. An account will be given below of the value of Green S as tracer dye in such rapidly excreted intramammary penicillin preparations. We have investigated whether the dye interferes with the antibiotic effect of the preparations *in vitro* and whether they irritate the glandular tissue. Further, we have studied the relation between the concentrations of tracer dye and penicillin in the milk produced.

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MATERIAL AND METHODS

As test animals, we used six cows, which were submitted to clinical and bacteriological examinations prior to the penicillin infusions. These examinations gave the following results:

Clinically: Quarter 3 of cow No. 35 and quarter 4 of cow No. 42 were slightly indurated. Quarter 3 of cow No. 37 was indurated and atrophic. The remaining quarters were clinically normal.

Bacteriologically: In a few of the quarters, "green" atypical streptococci were found now and again. In quarters 2 and 4 of cow No. 48 and in quarter 2 of cow No. 135, repeated examinations revealed infection with *Staphylococcus aureus*. The remaining quarters were bacteriologically normal.

The daily milk yield varied from 0.2 to 2.9 kg. per quarter, and the milk was found to be free from bacteriostatic substances before the infusion of the penicillin preparations. The infusions were given immediately after the evening milking, about 3 *p.m.*, and milk samples from the individual quarters thereafter drawn twice daily at the normal milking hours, *i.e.* about 7 *a.m.* and about 3 *p.m.*, for determination of the contents of penicillin and Green S.

Penicillin concentrations were determined by the agar-cup method, *Staph. aureus* 209 P and *Sarc. lutea* being used as test organisms, as indicated by *Simesen* (1959). Concentrations below 0.03 I.U./ml. were determined by the extraction technique indicated by *Hallas-Møller, Juncher, Møller & Wille* (1952). As, however, for practical reasons, we used quantities differing from those employed by these authors, our procedure is outlined below.

To 40 ml. milk, cooled to 1–2°C. by crushed ice, add 100 ml. of water-saturated butyl acetate and 24 ml. of 0.25 N-HCl, likewise ice-cooled. Shake the mixture vigorously for 1½ to 2 minutes. Leave the mixture in the ice bath until the milk phase has separated from the butyl acetate phase. Decant and measure the latter, and then shake it with 2 or 2.5 ml. phosphate-buffer (pH 8.0) for 1–2 minutes. In this way the penicillin contained in 40 ml. milk is transferred to 2 or 2.5 ml. buffer solution, in which the penicillin content is determined (agar-cup, *Sarc. lutea*). The sensitivity of this method is 0.002 I.U./ml.

The concentration of Green S in the milk was determined either after precipitation with acetone or by means of the ion-exchange technique described by *Rasmussen & Simesen* (1960). This last procedure mentioned needs the comment that reproducible results require shaking and analyses of prepared stan-

dards (0.05, 0.1 and 0.2 μ gm. dye/ml. milk) together with the unknown milksamples.

Experiments were carried through with six penicillin preparations to which Green S was added aseptically at the rate of 50 mg. per 100,000 I.U. of penicillin¹). The preparations were made up as follows:

- I. Procaine benzyl penicillin in a vegetable oil mixture of ointment consistency.
1 tube : 4.0 gm. 1 gm. : 25,000 I.U.
- II. Procaine benzyl penicillin in a vegetable oil mixture of ointment consistency, with polyoxyethylene sorbitan monoleate (Tween 80) added.
1 tube : 4.0 gm. 1 gm. : 25,000 I.U.
- III. Procaine benzyl penicillin in hydrophilic vegetable oil.
1 ml. : 25,000 I.U.
- IV. Procaine benzyl penicillin in hydrophilic vegetable oil, with polyoxyethylene sorbitan monoleate (Tween 80) added.
1 ml. : 25,000 I.U.
- V. Sodium benzyl penicillin dissolved in sterile, double-distilled water.
1 ml. : 50,000 I.U.
- VI. Sodium benzyl penicillin in a mixture of petroleum jelly and liquid paraffin, with polyoxyethylene sorbitan monostearate (Tween 60) and sorbitan monostearate (Span 60) added.
1 tube : 4.5 gm. 1 gm. : 25,000 I.U.

RESULTS

Influence of Green S on the antibiotic effect of penicillin

In preparations containing procaine benzyl penicillin, no destructive effect of Green S was demonstrable after storage at 20°C. for periods ranging from 2 weeks to 10 months. On the other hand, in prep. VI, sodium benzyl penicillin seemed to have been influenced to some extent by Green S, since, after about 9 months' storage, 47 per cent active penicillin was recovered in the preparation without Green S, against 24 per cent in the corresponding preparation with Green S. In the aqueous solution of sodium benzyl penicillin (prep. V) about 60 per cent active penicillin was recovered after 12 days at 4°C., both in the presence and in the absence of Green S.

¹) The preparations used were kindly prepared and placed at our disposal by *Novo Therapeutisk Laboratorium A/S*, Copenhagen.

Local irritating effect

To study the irritating effect of Green S on the glandular tissue we compared the reactions shown by the glands after intramammary infusion of penicillin preparations with and without Green S added. While palpation of the treated quarters revealed no signs of irritation, a macroscopic examination of the milk showed that preps. III and IV, both with and without Green S,

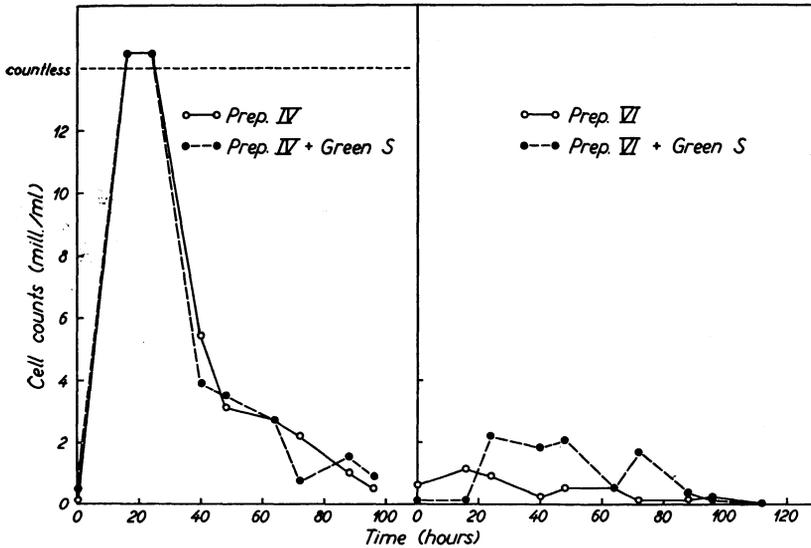


Fig. 1. Cell counts of the milk after infusion of procaine benzyl penicillin in hydrophilic vegetable oil with polyoxyethylene sorbitan monoleate added. Ordinate: Cell counts (mill./ml.). Abscissa: Hours after infusion.

Fig. 2. Cell counts in the milk after infusion of sodium benzyl penicillin dissolved in a mixture of petroleum jelly and liquid paraffin with polyoxyethylene sorbitan monostearate and sorbitan monostearate added. Ordinate: Cell counts (mill./ml.). Abscissa: Hours after infusion.

might produce clots at the first few milkings after the infusion. Cell counts on the milk showed that there was a very great difference between the irritating effects of the various preparations, regardless of whether they contained Green S or not. A preparation containing Green S generally gave a somewhat higher rise of the cell count than did the corresponding preparation without Green S, especially when the vehicle of the preparation had, in itself, no irritating effect. However, by the time the

excretion of penicillin and Green S had ceased, the degree of irritation, as indicated by the cell count, was of the same order. This will appear from Figs. 1 and 2, which show the cell counts of the milk during the first few days after infusion of preps. IV and VI, the ointment bases of which have the strongest and the weakest local irritating effect, respectively.

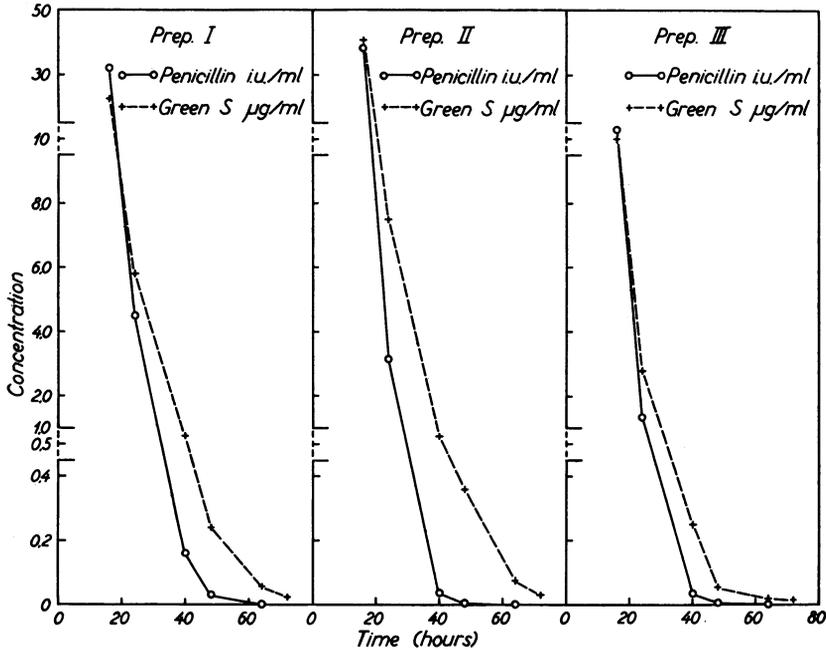


Fig. 3. Excretion in the milk of penicillin and Green S after infusion of preparations I, II, and III (100,000 I.U. penicillin, 50 mg. Green S). Ordinate: I.U./ml. of penicillin and $\mu\text{g.}/\text{ml.}$ of Green S. Abscissa: Hours after infusion.

Relation between concentrations of penicillin and Green S

After intramammary infusion of 100,000 I.U. of penicillin and 50 mg. Green S, the excretion of these components proceeded as shown in Figs. 3 and 4, in which all observations from a single experiment with each of the six preparations are rendered. In every case, a steep fall of the penicillin concentration to less than 0.1 I.U./ml. was observed within 40—48 hours. The concentration of Green S fell almost parallel to that of penicillin, and the excretion of both compounds was completed after about 90 hours,

										Preparation VI.										
										Preparation V.										
										Preparation IV.										
46	1	0.000	0.000	5	5	8	3	0.013	0.002	3	5	5	35	1	0.028	0.006	3	6	6	6
	2	0.030	0.000	4	5	8	4	0.056	0.020	3	5	5	2	2	0.034	0.005	4	6	6	6
	3	0.059	0.008	3	5	6	135	0.011	0.000	3	4	5	3	3	0.145	0.010	4	6	8	8
	4	0.123	0.015	3	5	5	2	0.010	0.000	3	4	5	4	4	1.010	0.053	2	6	6	6
max.		0.345	0.050				max.	0.160	0.035				37	1	0.104	0.005	3	6	6	6
aver.		0.112	0.009				aver.	0.045	0.006					2	0.180	0.006	3	7	8	8
min.		0.000	0.000				min.	0.000	0.000					3	0.420	0.052	3	7	8	8
	35	1	0.000	3	3	5	46	1	0.002	0.000	5	6	46	2	0.003	0.000	5	6	6	6
	2	0.013	0.003	3	5	5	3	0.002	0.000	5	6	6	4	4	0.002	0.000	5	6	7	7
	3	0.015	0.006	3	5	7	48	2	0.004	0.000	4	5	5	3	0.007	0.000	4	5	6	6
	4	0.072	0.005	3	5	7	4	0.002	0.000	4	5	7	4	3	0.003	0.000	4	5	5	5
37	1	0.014	0.000	3	4	8	46	1	0.034	0.007	3	5	46	1	0.067	0.004	4	6	6	6
	2	0.056	0.004	3	5	8	2	0.078	0.020	3	5	5	2	2	0.153	0.060	3	6	6	6
	3	0.003	0.000	4	5	8	3	0.049	0.008	3	5	6	3	3	0.420	0.052	3	7	8	8
	4	0.048	0.000	3	4	8	4	0.060	0.007	3	5	6	4	4	1.53	0.060	2	6	5	5
46	2	0.011	0.000	4	5	6	46	1	0.015	0.000	4	5	46	2	0.003	0.000	5	6	6	6
	4	0.010	0.000	3	4	5	3	0.009	0.000	4	5	6	4	4	0.002	0.000	5	6	7	7
	4	0.012	0.002	3	5	5	48	2	0.000	0.000	3	3	48	1	0.007	0.000	4	5	6	6
48	1	0.015	0.003	3	5	5	4	0.095	0.009	3	5	6	3	3	0.003	0.000	4	5	5	5
	3	0.015	0.003	3	5	5	4	0.059	0.002	4	6	8	46	1	0.067	0.004	4	6	6	6
46	1	0.004	0.000	5	6	8	46	1	0.059	0.002	4	6	46	1	0.067	0.004	4	6	6	6
	3	0.012	0.003	3	5	7	2	0.172	0.010	4	6	8	3	3	0.042	0.006	3	5	5	5
48	2	0.008	0.002	3	5	7	3	0.070	0.007	4	6	8	48	2	0.016	0.004	3	5	5	5
	4	0.000	0.000	4	5	8	4	0.134	0.008	4	6	8	48	2	0.036	0.002	3	5	5	5
48	2	0.003	0.000	4	5	6	48	1	0.006	0.000	4	5	46	2	0.002	0.000	5	6	8	8
	4	0.004	0.000	4	5	5	2	0.000	0.000	4	4	6	4	4	0.030	0.003	3	5	5	5
48	1	0.054	0.007	3	5	6	3	0.005	0.000	4	5	6	48	1	0.002	0.000	4	5	5	5
	3	0.014	0.002	3	5	7	4	0.016	0.000	4	5	7	3	3	0.004	0.000	4	5	5	5
max.		0.072	0.007				max.	0.172	0.020				max.		1.53	0.060				
aver.		0.018	0.002				aver.	0.041	0.004				aver.		0.178	0.011				
min.		0.000	0.000				min.	0.000	0.000				min.		0.002	0.000				

at the latest. The addition of a dispersing agent (preps. II, IV, and VI) seemed to influence neither the relation between Green S and penicillin concentrations nor the total excretion time.

To give a further illustration of the excretion of penicillin and tracer dye at the critical moment when the penicillin concentration is approaching zero, we have recorded, in Table 1, the

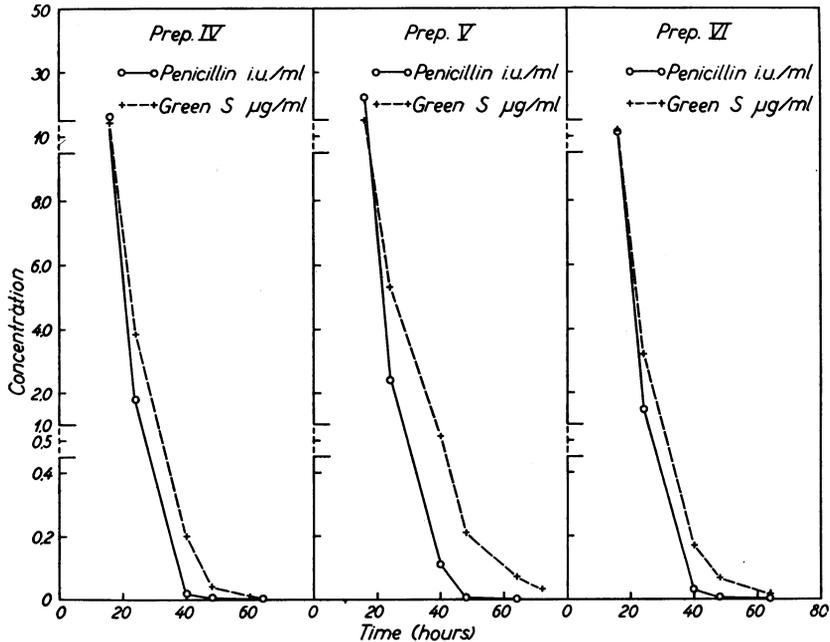


Fig. 4. Excretion in the milk of penicillin and Green S after infusion of preparations IV, V, and VI (100,000 I.U. penicillin, 50 mg. Green S). Ordinate: I.U./ml. of penicillin and $\mu\text{g.}/\text{ml.}$ of Green S. Abscissa: Hours after infusion.

concentrations of penicillin at, respectively, the last milking when the milk was still visibly discoloured, and the first milking when the green colour was no longer perceptible with the naked eye. In addition, the number is given of the last milking after the treatment when discoloration was observed directly and of the first milking at which the penicillin concentration was less than 0.002 I.U./ml. The last column shows how many milkings were required before the Green S content came below 0.03 $\mu\text{g.}/\text{ml.}$, which is the smallest amount measurable by the method indicated above.

The first column, in which the penicillin concentrations are recorded, shows that in 9 cases out of 112 the excretion of penicillin was concluded before the visible discoloration of the milk had disappeared. The remaining observations, especially in the experiments with preparations I and VI, showed considerable amounts of penicillin in the slightly green-coloured milk. At the following milking (next column) the penicillin concentrations were greatly reduced; in 49 cases the milk was apparently free from penicillin, and, apart from a single sample (II), concentrations were below 0.03 I.U./ml. in all experiments with preparations II, III, IV, and V. The highest penicillin concentration in milk of normal colour, *i.e.* 0.06 I.U./ml., was found in experiments with prep. VI.

From the columns indicating milking numbers it will be seen that, in the experiments with preps. II, III, IV, and V, all observations at the second milking after the last visible discoloration showed no penicillin, whereas in the experiments with preparations I and VI penicillin was traced in a few samples from the third and even the fourth milking after the last visible discoloration.

Regarding the time required for completion of the excretion of penicillin and Green S, respectively, (the last two columns) the greatest differences are seen in the experiments with preps. I and VI. In four cases (three with prep. I and one with prep. VI) no dye was detectable, although the milk contained penicillin. In the rest of the experiments, the excretion of Green S ceased simultaneously with or later than the excretion of demonstrable amounts of penicillin. In no instance was it possible, even by shaking the milk with an ion exchange resin, to trace the dye after the seventh milking, *i.e.*, about 90 hours after the infusion.

DISCUSSION

As pointed out by, among others, *Juncher, Magnusson & Rømer* (1950) and *Brown* (1960), the vehicle of an intramammary penicillin preparation plays an important part with regard to the excretion rate of the penicillin infused. In experiments with a preparation based on paraffin and liquid paraffin with polyethylene glycol sorbitan monostearate (Tween) added, *Rasmussen & Simesen* (1960) found a constant, short-term excretion, unlike in the cases of preparations containing no Tween, but aluminium monostearate. In the present investigations, in which

the vehicles used consisted of vegetable oil mixtures or hydrophilic vegetable oil, with and without polyoxyethylene sorbitan monoleate added, or of a mixture of petroleum jelly and liquid paraffin with polyoxyethylene sorbitan monostearate and sorbitan monostearate added, these dispersing agents were found to have no decisive influence on the excretion rate.

For all the preparations, the excretion time was of approximately the same length as for an aqueous solution of sodium benzyl penicillin. The maximum periods of excretion observed were 72 hours (preps. II, III, IV, and V) and 88 hours (preps. I and VI).

By addition of the tracer dye Edicol Supra Green B S (New C. I. 44090) to these ointment bases a satisfactory indication of the penicillin concentration in the milk produced is obtained. However, *Johns'* (1960) demand of "instant recognition and rejection" can only be met down to penicillin concentrations of maximum 0.06 I.U./ml. The last traces down to zero, which is the ideal that must be aimed at with a view to preventing allergic reactions in human consumers (cf. *Siegel* 1959; *Dalgaard-Mikkelsen* 1960; and others), are not accompanied by visible discoloration of the milk; these traces, however, can be disclosed by demonstrating the tracer dye by means of the ion-exchange technique indicated by *Rasmussen & Simesen* (1960). By this method, which can be carried through in practice within 15 minutes, it is possible to detect the dye until the milk contains less than 0.002 I.U./ml. of penicillin, provided a suitable ointment base has been used. It should therefore also be possible to secure penicillin-free milk. The fact that the tracer dye is in most cases traceable at one or two milkings after the penicillin concentration has fallen to below 0.002 I.U./ml. affords a further security against an even practically untraceable penicillin content in the milk.

The results of the present study give good reasons for paying careful attention to the vehicle, this having a marked influence on the length of the excretion time. The ideal is a vehicle which combines a short excretion time with a good therapeutic effect. In the latter respect, preparations with a short excretion time are, according to *Rømer* (1961), fully equal to those maintaining a prolonged penicillin excretion.

As Green S has no noteworthy influence, *in vitro*, on the antibiotic effect of the abovementioned rapidly excreted preparations,

and its local-irritating effect on the mammary tissue is of no practical importance, the addition of this substance to the preparations will afford a possibility of a quick and efficient control for penicillin in milk. This will mean a large step towards our aim: no penicillin in milk for consumption or industrial elaboration.

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SUMMARY

Green S (New C. I. 44090) has been added as tracer dye to six different, rapidly excreted intramammary penicillin preparations. 50 mg. Green S per 100,000 I.U. of penicillin did not affect the antibiotic effect of procaine benzyl penicillin preparations *in vitro*, whereas the antibiotic effect of sodium benzyl penicillin was somewhat reduced in preparations containing Green S, as compared to such without dye.

Great differences with regard to irritating effect was found between the various preparations, whether these contained Green S or not (Figs. 1 and 2). Preparations with Green S usually increased the cell count a little more than did the corresponding preparations with no Green S added, but by the time the excretion of penicillin had ceased, the irritating effect, as indicated by the cell count, was of the same order in both cases.

A direct relationship was demonstrated between the concentrations of penicillin and Green S in milk samples from six treated cows

(Figs. 3 and 4). Discoloration of the milk was recognizable with the naked eye at penicillin concentrations exceeding 0.06 I.U./ml. At penicillin concentrations from 0.06 to 0.002 I.U./ml. the tracer dye was readily detectable by means of an ion-exchange technique.

The excretion of the tracer dye in the milk being concluded simultaneously with, or one or two milkings after the penicillin concentration has fallen to below 0.002 I.U./ml. (Table 1), there should be a fair chance of avoiding penicillin in milk for consumption or industrial elaboration.

ZUSAMMENFASSUNG

Die Anzeigefarbe Green S (Food Green No. 4) in penicillinhaltigen Intramammaria. II.

Green S (New C. I. 44090) wurde 6 verschiedenen, schnell ausgeschiedenen penicillinhaltigen Intramammaria als Anzeigefarbe zugesetzt. 50 mg Green S pro 100.000 I.E. Penicillin beeinflusste nicht die antibiotische Aktivität von Prokain Benzylpenicillin Präparaten *in vitro*; dagegen wurde die antibiotische Wirkung von Natriumbenzylpenicillin-Präparaten in den Green S enthaltenden Zubereitungen etwas abgeschwächt im Vergleich zu den Präparationen ohne Farbstoff.

In der reizenden Wirkung der verschiedenen Präparate sowohl mit als auch ohne Zusatz von Green S wurde ein grosser Unterschied festgestellt (Fig. 1 und 2). Ein Zusatz von Green S ergibt in der Regel eine etwas grössere Zunahme der Zellenanzahl als das entsprechende Präparat ohne Green S, aber der an der Zellenzahl gemessene reizende Effekt ist von derselben Grössenordnung, wenn die Ausscheidung des Penicillins aufhört.

Zwischen den Konzentrationen von Penicillin und Green S in der Milch wurde bei 6 behandelten Kühen eine direkte Beziehung nachgewiesen (Fig. 3 und 4). Eine Verfärbung der Milch lässt sich direkt bei einem Penicillingehalt von über 0.06 I.E./ml erkennen. Bei einem Penicillingehalt von 0.06 I.E./ml bis zu 0.002 I.E./ml kann die Anzeigefarbe mit Hilfe der Ionenaustauschtechnik schnell nachgewiesen werden.

Da die Ausscheidung der Anzeigefarbe in der Milch gleichzeitig mit oder 1—2 Melkungen später zum Abschluss kommt, nachdem der Penicillingehalt unter 0.002 I.E./ml gesunken ist (Tabelle 1), wurde eine sehr gute Möglichkeit vorliegen, Penicillin in Milch zu vermeiden, die zum Konsum oder für die Produktion bestimmt ist.

SAMMENDRAG

Røbefarven Green S (Food Green No. 4) i penicillinholdige intramammaria. II.

Green S (New C. I. 44090) er tilsat 6 forskjellige, hurtigt udskilte penicillinholdige intrammaria som røbefarve. 50 mg Green S pr. 100.000 i.e. penicillin påvirkede ikke procain benzylpenicillin præ-

paraters antibiotiske aktivitet in vitro, mens natrium benzylpenicillinpræparaters antibiotiske aktivitet mindskedes noget mere i de præparationer, som indeholdt Green S, end i præparationer uden farvestof.

Der påvistes en stor forskel på den irriterende virkning af de forskellige præparater både med og uden tilsætning af Green S (Fig. 1 og 2). Tilsætning af Green S giver som regel en lidt større stigning i celletallet end det tilsvarende præparat uden Green S, men den irritative effekt målt ved celletallet er af samme størrelsesorden, når udskillelsen af penicillin ophører.

Der er påvist en direkte relation mellem koncentrationerne af penicillin og Green S i mælken fra 6 behandlede køer (Fig. 3 og 4). Misfarvning af mælken kan erkendes direkte ved et penicillinindhold på over 0.06 i.e./ml. Ved penicillinindhold fra 0.06 i.e./ml til 0.002 i.e./ml kan røbefarven hurtigt påvises ved hjælp af ionbytterteknik.

Da udskillelsen af røbefarven i mælken afsluttes samtidig med eller 1—2 malkninger efter, at penicillinindholdet er blevet mindre end 0.002 i.e./ml (Tabel 1), skulle der være særdeles god mulighed for at undgå penicillin i mælk til konsum eller produktion.

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