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TRACER DYES FOOD GREEN NO. 4 AND FOOD BLUE NO. 3 IN ANTIBIOTIC AND CHEMOTHERAPEUTIC PREPARATIONS FOR INTRAMAMMARY APPLICATION IV[1])

By

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In 1957 *Dalgaard-Mikkelsen & Rasmussen* reported that the addition of triphenylmethane dye Food Green No. 4 (New Colour Index 44090) to intramammary penicillin preparations would permit an indirect detection of penicillin in milk from the treated glands. This findings was subsequently confirmed by both Danish workers (*Rasmussen & Simesen* 1960; *Høgh & Rasmussen* 1961, 1962) and workers from other countries (*Kästli* 1962; *Müller* 1962; *Khalil* 1963). Studies have also been reported which show the relationship between concentrations of Food Green No. 4 and other antibiotics, e.g. chloramphenicol succinate in aqueous solution (*Wetli* 1963) and choramphenicol and tetracyclin-HCl in an ointment base (*Fritsche* 1964). *Shahani* (1961) successfully used Food Green No. 4 or Food Blue No. 3 (C.I. 42045) to detect indirectly various antibiotics in milk.

In the present work, which was encouraged by the great reduction in penicillin contamination of bulk milk resulting, under practical conditions, from the addition of Food Green No. 4 (*Høgh & Rasmussen* 1962) and Food Blue No. 2 *(Feagan* 1964) to intramammary penicillin preparations a comparison has been made between Food Green No. 4 and Food Blue No. 3 as tracer dyes for indirect detection in the milk of a number of antibiotic and chemotherapeutic compounds commonly used for intramammary treatment of cows with mastitis.

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MATERIALS AND METHODS

The experiments were carried out on 10 cows, which were subjected to clinical and bacteriological examination before application of the preparations in question. Quarter No. 4 of cow No. 42 was found to be slightly indurated, while the rest of the quarters were clinically normal. "Green" atypical streptococci were found occasionally in some quarters by the bacteriological examination, and quarter No. 4 of cow No. 48 was repeatedly found infected with *Staphylococcus aureus*. Otherwise all quarters were normal from the bacteriological point of view.

The daily milk yield varied from 3.5 to 13 kg per cow. The milk was free from bacteriostatic substances before application of the intramammary preparations. Infusions were given immediately after the afternoon milking at about 3 p.m. Milk samples from the individual quarters were taken twice daily at the normal milking hours, i.e. 7. a.m. and 3 p.m. The content of antibiotic or chemotherapeutic agent, and of Food Green No. 4 or Food Blue No. 3, was determined.

The content of penicillin was determined by the agar cup method with *Staph. aureus* 209 P and *Sarc. lutea* as test organisms, as indicated by *Simesen* (1959). Concentrations of less than 0.03 I.U./ml were determined by an extraction technique described by *Hallas-Møller et al.* (1952) and modified by *Høgh & Rasmussen* (1961) for determination of residues in milk. The sensitivity of the modified method is 0.002 I.U./ml milk. The content of oxytetracycline was measured with *Bac. cereus* ATCC 9634 as test organism, as indicated by *Grove & Randall* (1955). The sensitivity of this method is 0.1 μg/ml milk. The sulphonamide content was determined photometrically in a Coleman Jr. spectrophotometer after diazotizing and coupling, as indicated by *Bratton & Marshall* (1939). The method was modified by *Rasmussen* (1958) for use on milk. Its sensitivity is 1 μg/ml. In the experiments with the mixed preparation, Kefamycin®, only the penicillin content was determined.

In visibly discoloured milk, the amounts of Food Green No. 4 or Food Blue No. 3 were measured after precipitation with acetone, as described by *Rasmussen & Simesen* (1960) with regard to Food Green No. 4. Concentrations of tracer dye less than 0.2 to 0.3 μg/ml milk were determined by means of the ion exchange technique described by *Dalgaard-Mikkelsen & Rasmussen* (1962). The sensitivity of this rapid method is 0.02 μg/ml.

Experiments were carried out with six different intramammary preparations. Details of declaration, dosage and tracer-dye addition are given below:

I: Penicillin procaine Novo vet.®
1 ml: 25,000 I.U. Dose: 4 ml per quarter.
Food Green No. 4 or Food Blue No. 3: 50 mg/dose.

II: Leocillin vet.® susp. (Penetamate hydroiodide).
1 ml: penicillin ester corresponding to 250,000 I.U. penicillin. Dose: 2 ml per quarter.
Food Green No. 4 or Food Blue No. 3: 50 mg/dose.

III: Kefamycin® susp.
1 ml: 20,000 I.U. potassium penicillin, 250 U. bacitracin and 50 mg neomycin sulphate.
Dose: 5 ml per quarter.
Food Green No. 4 or Food Blue No. 3: 50 mg/dose.

IV: Solutio Terramycin vet.®
1 ml: 30 mg oxytetracycline. Dose: 10 ml per quarter.
Food Green No. 4 or Food Blue No. 3: 50 mg/dose.

V: Supronal emulsion 20 % ad us.vet.®
Equal portions sulphamerazine (NFN) and sulphatolamide (NFN).
1 ml: 200 mg. Dose: 40 ml per quarter.
Food Green No. 4 or Food Blue No. 3: 50 mg/dose.

VI: Vetisulid® suspension.
Sulphachlorpyridazine (NFN).
1 ml: 50 mg. Dose: 30 ml per quarter.
Food Green No. 4: 25 mg/dose.

RESULTS

Stability of the preparations.

The amounts of therapeutic agent and tracer dye in preparations containing Food Green No. 4 or Food Blue No. 3 were determined after storage at room temperature. In preparations I, II, III, V, and VI drugs as well as tracer dyes proved very stable; up to six months after the preparations were produced 75—100 per cent of the drugs and 70—100 per cent of the tracer dyes could be recovered. The dyes had no effect on the stability of the penetamate hydroiodide (preparation II), since less than 1 per cent of the penicillin ester was hydrolyzed. In preparation IV, after storage at 4°C and 20°C for two months, 70—100 per cent of the oxytetracycline content could be recovered whether or not Food Blue No. 3 or Food Green No. 4 had been added. The content of the tracer dyes was at the same time recovered by 40—80 per cent.

Local irritating effect.

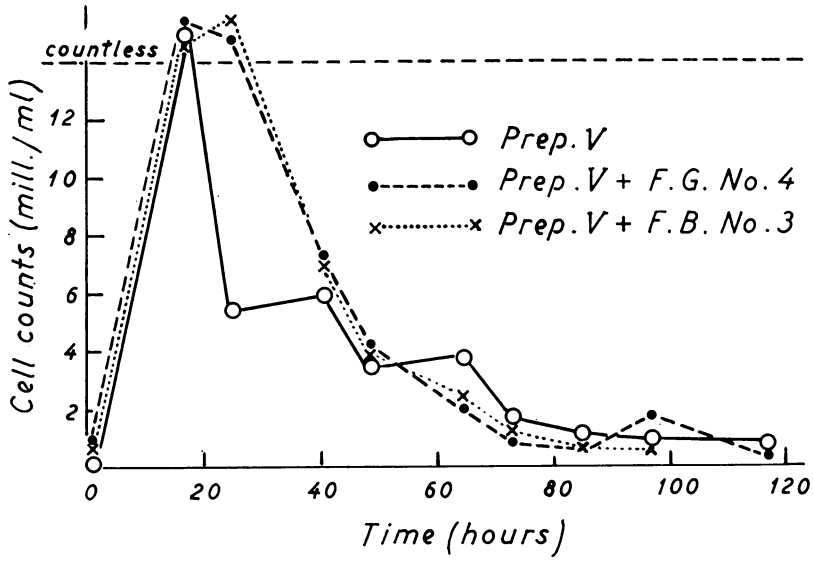
It has been shown previously (*Høgh & Rasmussen* 1961) that the addition of Food Green No. 4 to intramammary preparations did not alter the local irritating effect on the glands. In order to compare Food Green No. 4 and Food Blue No. 3 from this point of view, the number of cells in the milk was counted after intramammary application of preparations with and without tracer dyes. Judged by the cell counts, there were great variations in the irritating effect of the preparations, whether or not tracer dye had been added. Fig. 1 shows the cell counts of the milk during the first days after treatment with preparation V. At the first milkings after the treatment, the cell count was considerably increased, but it was normal again at the sixth milking. The addition of tracer dyes does not appear to increase the irritating effect of the preparations.

Relationship between concentrations of therapeutics and tracer dyes in milk.

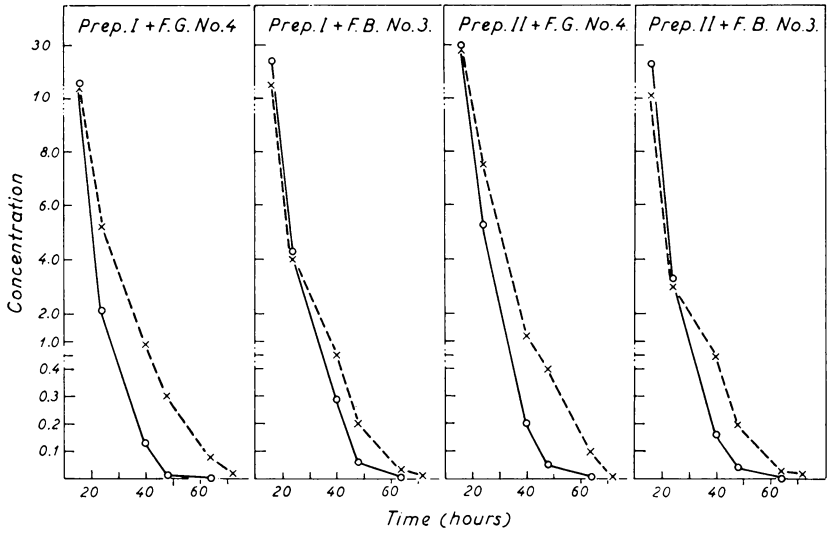
The concentrations of drugs and tracer dyes found in the milk after intramammary infusion will appear from Figs. 2—4, in which all analytic results are given from one experiment with each of the six preparations to which Food Green No. 4 or Food Blue No. 3 were added.

With all the preparations containing penicillin, the content of this drug in the milk was rapidly reduced through the first milkings after the application; after 40—48 hours it was less than 0.1 I.U./ml (Figs. 2 and 3). Excretion of oxytetracycline also occurred very quickly, the concentration being less than 0.1 μg/ml 64 to 72 hours after application (Fig. 3). The decrease in the amounts of Food Green No. 4 as well as of Food Blue No. 3 was parallel to the decrease in the content of penicillin, penicillin ester and oxytetracycline, and in all cases the excretion both of antibiotics and tracer dyes was completed about 90 hours after application.

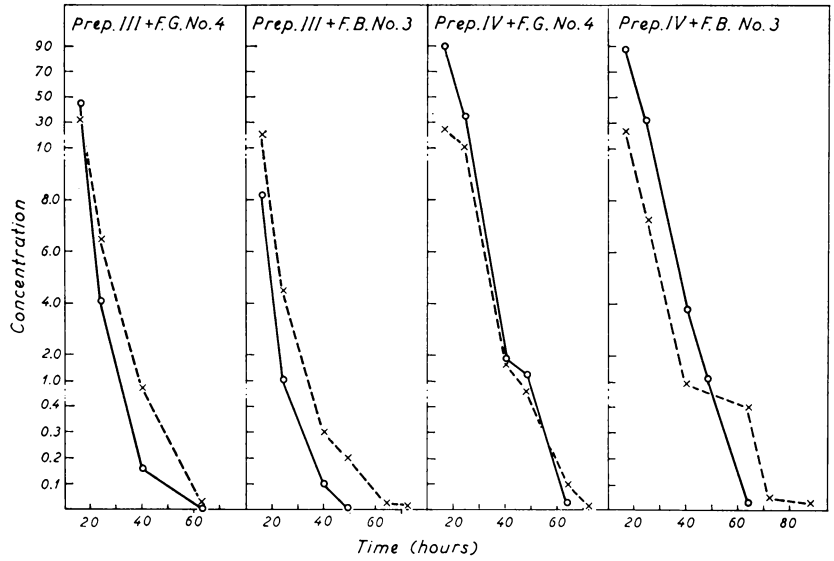
The excretion of sulphonamides and tracer dyes is shown in Fig. 4. The content of both substances was reduced very considerably through the first milking. The initial concentration of sulphonamide was greater with Supronal than with Vetisulid. This is due to the dose of Supronal (40 ml, 20 per cent) being larger than that of Vetisulid (30 ml, 5 per cent). This difference



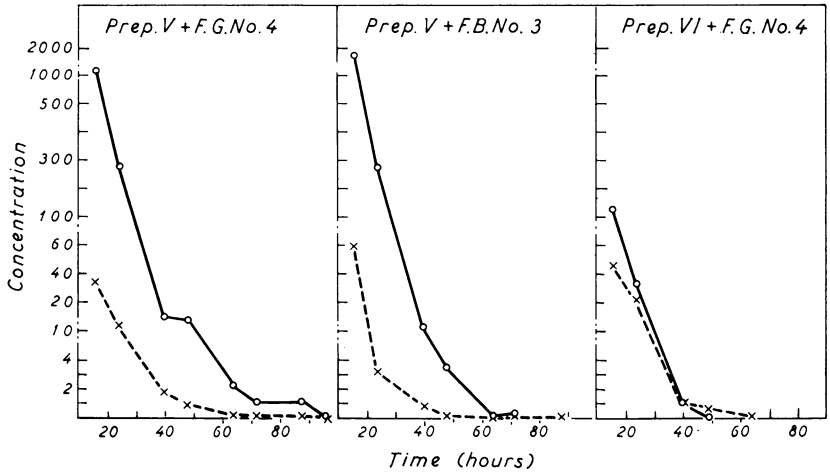
F i g. 1. Cell counts in milk after infusion of Supronal emulsion 20 % ad us.vet.® Ordinate: Cell counts (mill./ml). Abscissa: Hours after infusion.



F i g. 2. Excretion in milk of penicillin (o———o) and tracer dye (x------x) after infusion of preparations I and II. Ordinate: I.U./ml of penicillin and μg/ml of tracer dye. Abscissa: Hours after infusion.



F i g 3. Excretion in milk of penicillin or oxytetracycline (o———o) and tracer dye (x------x) after infusion of preparations III and IV. Ordinate: I.U./ml of penicillin or μg/ml of oxytetracycline and tracer dye. Abscissa: Hours after infusion.



F i g. 4. Excretion in milk of sulphonamide (o——o) and tracer dye (x------x) after infusion of preparations V and VI. Ordinate: μg/ml of sulphonamide and tracer dye. Abscissa: Hours after infusion.

in dosage probably also explains the fact that with Supronal the curves run higher for sulphonamide than for the tracer dyes, while the curves for sulphonamide and tracer dye are almost identical after application of Vetisulid. The elimination of Vetisulid and Food Green No. 4 was complete, respectively, 48 to 64 hours and 80 to 96 hours after application.

The time required for complete elimination of drugs and tracer dyes are further illustrated by the data recorded in Table 1. The table shows, for each individual quarter, the number of milkings following application at which the milk was visibly discoloured, and the first milking at which, respectively, the content of penicillin was less than 0.002 I.U./ml, the content of oxytetracycline less than 0.1 μg/ml, the content of sulphonamide less than 1 μg/ml, and the content of tracer dye less than 0.02 μg/ml.

It will be seen that in all cases the milk was visibly discoloured at up to two milkings before the excretion of antibiotics and chemotherapeutics ceased. By using the sensitive ion exchange method for detection of the tracer dyes, these were demonstrable at least as long as and often one or two milkings longer than the drugs.

Recovery of drugs and tracer dyes in the milk.

A calculation was made of the total amounts of drugs and tracer dyes excreted in the milk of the experimental cows. As will appear from Table 2, there were very great variations in the recovery rates both of drugs and of dyes. The highest rates were observed in the highest-yielding cows. There were also great variations in the recovery of the same drug from different quarters, and in the recovery of different drugs from identical quarters. The amounts excreted of Leocillin, Supronal and Vetisulid (preparations II, V, and VI) were less than the amounts excreted of Penicillin procaine, Kefamycin and Terramycin (preparations I, III and IV). The recovery of the two tracer dyes varied considerably, irrespective of the preparation in which they were incorporated.

Absorption from the mammary gland.

The drugs could be demonstrated in the blood up to 24 hours after intramammary application. The tracer dyes could not be

Table 1.

Milking number at which the excretion of drugs and tracer dyes was concluded.

Cow No.	Quarter No.	Food Green No. 4 added: Last discoloured milk sample (Milking No.)	Food Green No. 4 added: Penicillin conc. lower than 0.002 I.U./ml.	Food Green No. 4 added: Dye conc. lower than 0.02 µg/ml.	Cow No.	Quarter No.	Food Blue No. 3 added: Last discoloured milk sample (Milking No.)	Food Blue No. 3 added: Penicillin conc. lower than 0.002 I.U./ml.	Food Blue No. 3 added: Dye conc. lower than 0.02 µg/ml.
Preparation I									
46	1	5	6	7	28	1	3	6	7
	2	4	6	7		2	2	5	6
	3	3	5	5		3	4	7	8
	4	3	5	5		4	4	7	8
48	1	3	4	7	48	1	3	5	6
	2	3	5	5		2	3	5	6
	3	5	5	8		3	3	5	6
	4	3	5	5		4	3	5	6
46	1	4	5	5	26	1	3	5	6
	2	5	6	7		2	3	5	6
	3	3	4	5		3	3	6	7
	4	3	5	7		4	3	5	6
max.		5	6	8			4	7	8
min.		3	4	5			2	5	6
Preparation II									
26	1	4	6	6	26	1	2	5	5
	2	3	5	5		2	2	5	5
	3	4	6	6		3	3	6	7
	4	4	6	7		4	2	5	6
42	1	4	6	6	42	1	3	7	7
	2	4	5	6		2	3	6	7
	3	4	5	6		3	3	5	6
	4	3	5	6		4	2	5	5
48	1	4	6	6	48	1	2	5	5
	2	3	5	6		2	2	4	6
	3	4	5	7		3	2	5	6
	4	3	5	6		4	2	5	7
max.		4	6	7			3	7	7
min.		3	5	5			2	4	5
Preparation III									
48	1	3	4	8	26	1	3	6	7
	2	3	4	7		2	3	6	7
	3	4	6	8		3	4	6	7
	4	3	4	6		4	3	6	7
42	1	3	4	7	51	1	3	4	6
	2	2	4	6		2	3	4	5
	3	2	4	6		3	2	5	5
	4	3	4	6		4	3	5	6
1	1	2	5	6	43	1	3	6	8
	2	2	5	6		2	2	6	7
	3	3	5	8		3	3	6	7
	4	3	5	8		4	3	6	8
max.		4	6	8			4	6	8
min.		2	4	6			2	4	5

Table 1 (continued).

Preparation IV

Cow No.	Quarter No.	Food Green No. 4 added: Last discoloured milk sample (Milking No.)	Food Green No. 4 added: Oxytetracycline conc. lower than 0.1 μg/ml.	Food Green No. 4 added: Dye conc. lower than 0.02 μg/ml.	Cow No.	Quarter No.	Food Blue No. 3 added: Last discoloured milk sample (Milking No.)	Food Blue No. 3 added: Oxytetracycline conc. lower than 0.1 μg/ml.	Food Blue No. 3 added: Dye conc. lower than 0.02 μg/ml.
26	1	4	5	6	26	1	3	6	7
	2	3	5	6		2	3	6	7
	3	4	5	6		3	4	6	7
	4	4	6	6		4	4	6	7
42	1	4	5	6	42	1	4	6	7
	2	3	5	6		2	3	5	5
	3	3	5	6		3	4	5	7
	4	3	5	6		4	3	5	6
59	1	3	6	6	59	1	4	6	6
	2	3	6	6		2	4	7	7
	3	3	6	6		3	3	6	6
	4	3	6	6		4	4	6	6
max.		4	4	6			4	7	7
min.		3	5				3	5	5

Preparation V

Cow No.	Quarter No.	Last discoloured milk sample	Sulphonamide conc. lower than 1 μg/ml.	Dye conc. lower than 0.02 μg/ml.	Cow No.	Quarter No.	Last discoloured milk sample	Sulphonamide conc. lower than 1 μg/ml.	Dye conc. lower than 0.02 μg/ml.
15	2	5	6	9	15	2	3	5	9
	3	5	8	9		3	3	5	9
48	2	3	5	9	127	2	3	5	9
	3	6	11	11		3	3	5	9
59	1	4	7	7	59	2	3	6	6
	4	4	8	7		3	3	5	6
max.		6	11	11				5	9
min.		3	5	7				4	6

Preparation VI

70	1	4	4	8
	2	4	4	8
	3	4	4	8
	4	4	4	8
15	1	4	4	7
	2	4	4	7
	3	4	4	7
	4	4	4	7
70	1	5	4	8
	2	4	4	7
	3	4	5	7
	4	4	5	7
max.		5	5	8
min.		4	4	7

Table 2. Recovery of drugs and tracer dyes after intramammary application.

Preparation No.	Drug recovery			Food Green No. 4 recovery			Food Blue No. 3 recovery		
	min. %	max. %	average %	min. %	max. %	average %	min. %	max. %	average %
I	5	34	21	24	54	40	11	40	26
II	3	25	10	26	95	77	17	52	32
III	5	87	24	14	74	44	20	75	50
IV	24	88	45	30	92	70	34	99	70
V	4	31	11	31	78	51	27	62	42
VI	1	3	2	30	44	39	—	—	—

demonstrated in the blood, but were found in the urine during the first 24 hours after application. The urine of three cows, taken with balloon catheter inserted in the bladder, contained 5—15 mg Food Green No. 4 or Food Blue No. 3, corresponding to 2.5 to 7.5 per cent of the amount infused into the udder by intramammary infusion (4×50 mg).

DISCUSSION

In all quarter samples tracer dye was demonstrable for at least as long as the drugs, and most often longer. The experiments have thus proved the great certainty with which triphenylmethane tracer dyes can reveal, indirectly, the presence of antibiotics and sulphonamides in the milk after intramammary application.

It was found in previous investigations that 50 mg Food Green No. 4 is a suitable amount of tracer dye per 100,000 I.U. penicillin (*Dalgaard-Mikkelsen & Rasmussen* 1957; *Rasmussen & Simesen* 1960; *Høgh & Rasmussen* 1961, 1962; *Khalil* 1963). Also in the present study 50 mg tracer dye per dose of the various preparations was found suitable, except for Vetisulid, for which 25 mg per dose was sufficient. The fact that a lesser amount of tracer dye is needed per dose of Vetisulid must be viewed in relation to the small amount of sulphonamide per dose of Vetisulid as compared to the amount per dose of Supronal.

Further, the present study has shown that 50 mg tracer dye is sufficient to demonstrate residues of terramycin after intramammary application of 10 ml Sol. Terramycin vet. 3 per cent

(300 mg). Also *Shahani* (1961) has reported that either Food Blue No. 3 or Food Green No. 4 is suitable for demonstration of oxytetracycline, but unfortunately this writer has made no mention of the amounts of oxytetracycline and tracer dye, nor of the type of vehicle. *Fritsche* (1964) found that 50 mg Food Green No. 4 per 500 mg tetracycline HCl in an oil and fat containing base was insufficient for direct visual demonstration of tetracycline residues. On the other hand, *Fritsche* (1964) and *Wetli* (1963) recommended 50 mg Food Green No. 4 per 500 mg chloramphenicol in oil containing vehicles or water. From the concentrations recorded in *Fritsche*'s work (1964), it appears that the rate of recovery (in the milk) is higher for tetracycline than for chloramphenicol, and it is possible, therefore, that by the use of a larger amount of tracer dye or a more sensitive method for its demonstration (ion exchange method) also residues of tetracycline HCl, administered by the intramammary route, in oil containing vehicles might be demonstrated effectively with Food Green No. 4.

Rates of recovery for drugs and tracer dyes given by the intramammary route vary considerably (Table 2). According to the literature, recovery is primarily dependent on the milk yield, a large milk yield often giving a high recovery, while the opposite is the case when the daily milk yield is poor. Other factors, however, such as type of vehicle and rate of absorption from the mammary gland tissue may also influence the recovery (see *Khalil* 1963). Despite the varying recovery rates recorded for each individual preparation, Table 2 shows that the recovery values are highest for penicillin and oxytetracycline (preparations I, III and IV) and lowest for the penicillin ester and the two sulphonamide preparations (II, V and VI). This is probably due to difference in the ability of the drugs to penetrate the epithelium of the mammary glands. As shown by *Rasmussen* (1962) the degree of ionization and lipid solubility of the drug influence the absorption from the mammary gland, drugs which are generally found in molecular form and which have a large lipid solubility coefficient being absorbed rapidly and to a larger extent than drugs which are ionized and have a low lipid solubility coefficient.

The recovery of both Food Green No. 4 and Food Blue No. 3 which also varies considerably (Table 2), corresponds to what was found by *Khalil* (1963) as regards Food Green No. 4 and by

Dawson & Feagan (1960) as regards Food Blue No. 2 in intramammary penicillin preparations. In contrasts to *Khalil* (1963), who states that Food Green No. 4 is not absorbed from the milk gland, the present study shows that Food Green No. 4 is absorbed, since tracer dye can be demonstrated in the urine the first day after intramammary application.

The tracer dyes, Food Green No. 4 and Food Blue No. 3 and No. 2 seem equally suitable for practical use. Therefore, as mentioned by *Dalgaard-Mikkelsen & Rasmussen* (1962) a choice may be made between the three dyes, according to what is permitted by the health authorities of the different countries. An evaluation of the toxicity of these tracer dyes, as given f. inst. in the priority list published (1964) by the British Ministry of Agriculture, Fisheries and Food, has placed Food Green No. 4 most favourably.

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SUMMARY

Comparison has been made between Food Green No. 4 and Food Blue No. 3 as tracer dyes for indirect detection of the drugs in milk after intramammary application of a number of commonly used preparations containing antibiotics and chemotherapeutic agents.

The tracer dyes had no effect on the stability of the preparations containing penicillin, penicillin ester, and sulphonamide (I, II, III, V and VI); after six months' storage at room temperature, 75 to 100 per cent of the drugs could be recovered. The oxytetracycline content in preparation IV recovered by 70 to 100 per cent after two months. There were great differences in the irritating effect of the various preparations, as determined by the cell counts of the milk, but the addition of tracer dye did not alter this effect. A direct relationship was demonstrated between concentrations of, on the one hand, tracer dye and, on the other hand, penicillin (preparations I, II, and III),

oxytetracycline (preparation IV) and sulphonamide (preparations V and VI) in milk from ten treated cows.

In all quarter samples tracer dye could be demonstrated for just as long as the drugs, and often one or two milkings longer. Thus the experiments confirm the great certainty with which triphenylmethane dyes can reveal indirectly the presence of very small residues of antibiotics and sulphonamides in the milk after intramammary infusion.

ZUSAMMENFASSUNG

Die Indikatorfarbstoffe Food Green No. 4 und Food Blue No. 3 in Antibiotika- und Chemotherapeutika-enthaltenden Intramammaria, IV.

Ein Vergleich zwischen Food Green No. 4 und Food Blue No. 3 als Indikatorfarbstoffe zum indirekten Nachweis von Arzneimitteln in der Milch nach intramammärer Infusion mit einer Reihe gewöhnlich benutzten Antibiotika- und Chemotherapeutika-enthaltenden Intramammaria ist vorgenommen worden.

Die Indikatorfarben beeinflussten nicht die Aktivität der Penicillin-, Penicillinester- und Sulfonamid-haltigen Präparate (I, II, III, V und VI). Nach einer 6monatigen Aufbewahrung bei Zimmertemperatur konnte 75 bis 100 % der Arzneimittel wiedergefunden werden. Nach dem Verlauf von 2 Monaten konnte 70 bis 100 % des Oxytetracyclingehaltes in Präparation IV wiedergefunden werden. Die irritierende Wirkung der verschiedenen Präparate war sehr unterschiedlich. Jedoch änderte der Zusatz von Indikatorfarben nicht den irritativen Effekt gemessen an der Zellenanzahl in der Milch. Es konnte ein direkter Zusammenhang zwischen der Konzentration von Indikatorfarbstoff und den Konzentrationen von Penicillin (die Präparate I, II und III), Oxytetracyclin (Präparat IV) und Sulfonamid (die Präparate V und VI) in der Milch von zehn behandelten Kühen festgestellt werden.

Da in den Milchproben von allen Einzeldrüsen Indikatorfarbstoff durch ebenso lange Zeit und oft sogar etwas länger als die Arzneimittel festgestellt werden konnte, haben die Versuche die grosse Sicherheit bestätigen können mit der die Triphenylmethan-Farbstoffe indirekt die Anwesenheit von minimalen Antibiotika- und Sulfonamid-Mengen in der Milch nach intramammärer Applikation anzeigen.

SAMMENDRAG

Røbefarverne Food Green No. 4 og Food Blue No. 3 i antibiotika- og kemoterapeutikaholdige intramammaria, IV.

Der er foretaget en sammenligning mellem Food Green No. 4 og Food Blue No. 3 som røbefarver til indirekte påvisning af lægemidler i mælken efter intramammær infusion af en række almindeligt benyttede antibiotika- og kemoterapeutikaholdige intramammaria.

Røbefarverne påvirkede ikke de penicillin-, penicillinester- og sulfonamidholdige præparaters (I, II, III, V og VI) aktivitet, idet der

efter 6 måneders opbevaring ved stuetemperatur genfandtes 75—100 % af lægemidlerne. Efter 2 måneders forløb genfandtes 70—100 % af oxytetracyklinindholdet i præparation IV. Der påvistes stor forskel på den irriterende virkning af de forskellige præparater, men tilsætning af røbefarver ændrede ikke præparaternes irritative effekt målt ved celletallet i mælken. Der er påvist en direkte relation mellem røbefarvernes koncentration og koncentrationerne af penicillin (præparaterne I, II og III), oxytetracyklin (præparat IV) og sulfonamid (præparaterne V og VI) i mælken fra 10 behandlede køer.

Da der i alle enkeltkirtelprøverne er påvist røbefarve i mælken i lige så lang tid og oftest 1—2 malkninger længere end lægemidlerne, har forsøgene bekræftet den store sikkerhed, hvormed triphenylmethan farvestofferne indirekte kan røbe tilstedeværelsen af minimale rester af antibiotika og sulfonamider i mælken efter intramammær applikation.

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