Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 10, *Number* 2, *April*, 1938.

Printed in the Union of South Africa by the Government Printer, Pretoria.

Quantitative Studies upon Porphyrin Excretion in Bovine Congenital Porphyrinuria (Pink Tooth) No. I.

By CLAUDE RIMINGTON and G. C. S. ROETS, Section of Toxicological Chemistry; and P. J. J. FOURIE, Section of Hygiene, Onderstepoort.

THE occurrence of congenital porphyrinuria in a bovine herd has been recently reported (Fourie 1936, Fourie and Rimington 1937) and a study made of the types of porphyrin present in these cases Rimington 1936 and 1937, Rimington and Roets 1937).

Since the literature reveals little accurate information as to the quantities of porphyrins excreted in congenital porphyrinuria, studies of these bovine cases have been undertaken with a view to ascertaining whether or not fluctuations occurred in the quantities of copro- and uroporphyrin eliminated with the urine and faeces and if any correlation was to be observed between such data as urine volume and porphyrin output. Not only was it hoped that the results would contribute materially to the elucidation of the disease, considered as a derangement of endogenous pigment metabolism, but we were also anxious to throw further light upon the uneven distribution of porphyrin in the bones of affected animals. The case previously slaughtered for examination showed the bone structure, in transverse section, to be stained unevenly in rings of darker and lighter colour (compare Fink and Hoerburger 1935).

HISTORICAL.

It must be stated at the outset that the distinction usually drawn between congenital (including the so-called chronic cases) and acute idiopathic porphyrinuria is by no means a wholly satisfactory one. Thus, in the former type, excretion of porphyrin is generally regarded as being practically continuous from birth (congenital) or sometime after (chronic) whilst in the acute form of the disease porphyrin excretion is paroxysmal. Similarly the pigments of the congenital type belong mainly to the I series (but compare Fischer and Hofmann 1937, Rimington 1936, 1937) whilst those of acute porphyrinuria are preponderately series III derivatives (Waldenström 1935, Mertens 1936, Dobiner 1936). Transition forms between the " chronic " and " acute " types of the disease are occasionally to be encountered (van den Bergh, Regniers and Muller 1928, van den Berg and Grotepass 1937 and Dobiner 1936).

Prior to Hans Fischer's investigation of the "Petry" case and isolation of copro- and uroporphyrin, workers in this field were wont to refer to the mixture of pigments in urine or faeces as "haematoporphyrin". The methods they used for quantitative determination were crude (see Garrod 1923, Günther 1911, 1922) and no very great reliance can be placed upon their figures. Nevertheless Garrod mentions cases in which pigment excretion was intermittent and thus like that of Arzt and Hausmann (1920) in which the colour of the urine varied appreciably at different periods and this has also been the experience of other workers. Fischer estimated that Petry excreted about 0·4 gm. total porphyrin per day.

Quantitative investigations using refined methods of analysis have recently been carried out upon copro-porphyrin III excretion in lead poisoning (Mertens 1937) in which case it was found that there was a definite tendency for urinary porphyrin and urine volume to run a parallel course. From Dobiner's recent work (1937a; 1937b) upon porphyrin excretion in various pathological states it is difficult to tell whether any such regularity occurred. He states that the daily copro- and uroporphyrin excretion varied considerably but calculating daily averages over 3 to 10 day periods of observations, the figures for these periods were comparable.

Methods.

The animals were kept in metabolism stables provided with a channel for collecting the urine in a glass receptacle and in order to minimise possible contamination with faecal material, assistants were employed day and night whose duty it was to shovel up the faeces directly they were passed into weighed tins. The animals were maintained upon the usual laboratory ration and watered from a bucket twice daily. Collection extended over 5 to 6 day periods and each daily batch of material was worked up immediately.

Urines.—The volume was measured and an aliquot of 150 to 300 c.c. (according to porphyrin content) acidified with acetic acid to a final concentration of 5 per cent. and the mixture then extracted with ether for 24 hours in a Kutscher-Steudel continuous extraction apparatus. All ether-soluble porphyrin was in this way removed and troublesome emulsions avoided. The ether extract was washed in a separatory funnel with water containing potassium acetate until the bulk of the acetic acid had been removed. The water washings usually contained urobilin or a similar pigment. The total porphyrin was now transferred, by shaking, with 5 per cent. hydrochloric acid and this solution made up to a convenient volume. An aliquot usually 5 c.c. was then placed in a test tube and diluted with water until the intensity of the absorption band at 550 matched that of a standard solution, similarly observed by means of a pocket spectroscope, and containing 1 mg. coproporphyrin in 100 c.c. of 5 per cent. hydrochloric acid. With practice small differences between test and standard solutions could easily be detected but we put the error of the whole determination on the conservative side as ±10 per cent. Colorimetric comparison of the acid solutions would have been possible and gave in check experiments nearly identical results, but

we preferred to base our determinations upon a specific character such as the intensity of an absorption band rather than on anything so unspecific as colour.

Uroporphyrin was determined in the ether extracted urine by an analogous process after conversion into the more stable copper complex. In preliminary experiments an endeavour was made to purify and concentrate the pigment by filtering it through adsorption columns of alumina and then eluting with diluted alkali but this procedure proved both troublesome and time-consuming. Finally, direct determinations were made as follows: The ether-extracted urine was transferred to a 200 or 500 c.c. graduated flask and made up to the neck with a 5 per cent. sodium hydroxide, the container and spiral of the Kutscher-Steudel apparatus being rinsed out with the same solution. The final reaction of the mixture was alkaline and a copious micro crystalline precipitate separated leaving a clear yellow-brown supernatant liquid. An aliquot of 10 c.c. of this was transferred to a 50 c.c. flask, two drops of saturated aqueous copper acetate solution added and the mixture digested on a warm plate for five minutes using a lightly fitting glass stopper as a hang-in condensor to prevent loss of liquid by evaporation. The reaction mixture was then filtered, a 5 c.c. aliquot placed in a small test tube and the intensity of the 569·5 band of the uroporphyrin copper complex spectrum compared with that of a standard prepared from pure uroporphyrin and copper acetate. A series of tubes was kept containing dilutions of the standard equivalent to 1, 1·2, 1·4, 1·6, 1·8 and 2·0 mgm. pigment per 100 c.c. and the concentration in the unknown estimated to the nearest tenth. Thus an unknown stronger than the 1·6 but weaker than the 1·8 standard was said to contain 1·7 mgm. per 100 c.c. Admittedly the error of determination is here again about ± 10 per cent. but the method was rapid. No other method is known permitting of the quantitative determination of uroporphyrin in such complex mixtures with any higher degree of accuracy. Separation from the accompanying brown and yellow pigments of the urine requires an elaborate technique and inevitably entails serious losses. When determination is based upon the intensity of an absorption band, simultaneous presence of other non-specific absorbing pigments has but a negligible effect.

Faeces.—The quantity of coproporphyrin excreted per rectum was determined as follows: The entire faecal mass passed during 24 hours was mixed as intimately as possible and after weighing an aliquot of 100 gms. was subjected to the usual extraction technique with acetic acid and ether. The ether-soluble porphyrin was finally passed into 5 per cent. hydrochloric acid, this solution shaken with chloroform if necessary and the intensity of the 550 absorption band matched against the standard as previously described. Duplicate determinations upon both faeces and urine yielded consistent results.

Results.

A summary of the numerical results is presented in tables and also graphically in the diagrams Figs. 1 and 2.

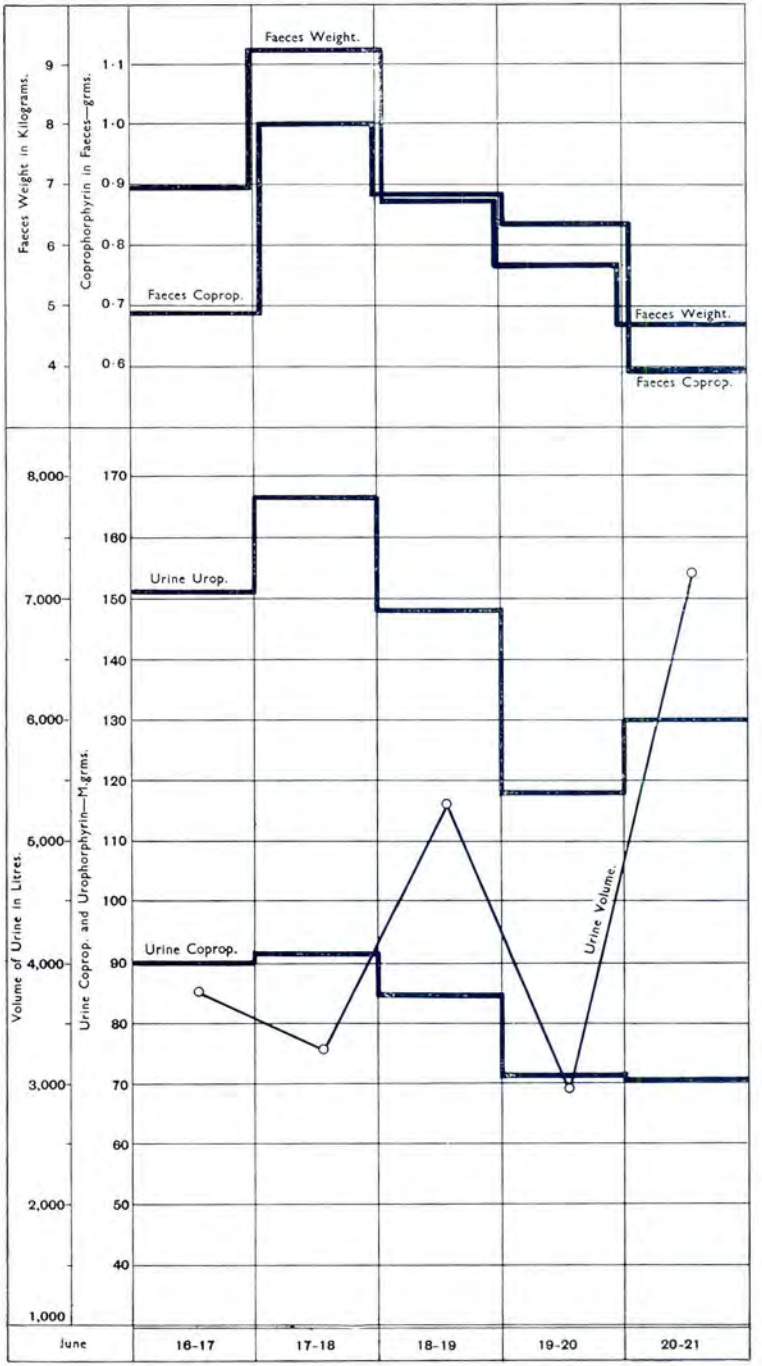
Faeces Weight in Kilograms.
Coprophorphyrin in Faeces—grms.
Faeces Weight.
Faeces Coprop.
Faeces Weight.
Faeces Coprop.
Volume of Urine in Litres.
Urine Coprop. and Urophorphyrin—M.grms.
Urine Urop.
Urine Coprop.
Urine Volume.
June
16-17
17-18
18-19
19-20
20-21

Fig. I.

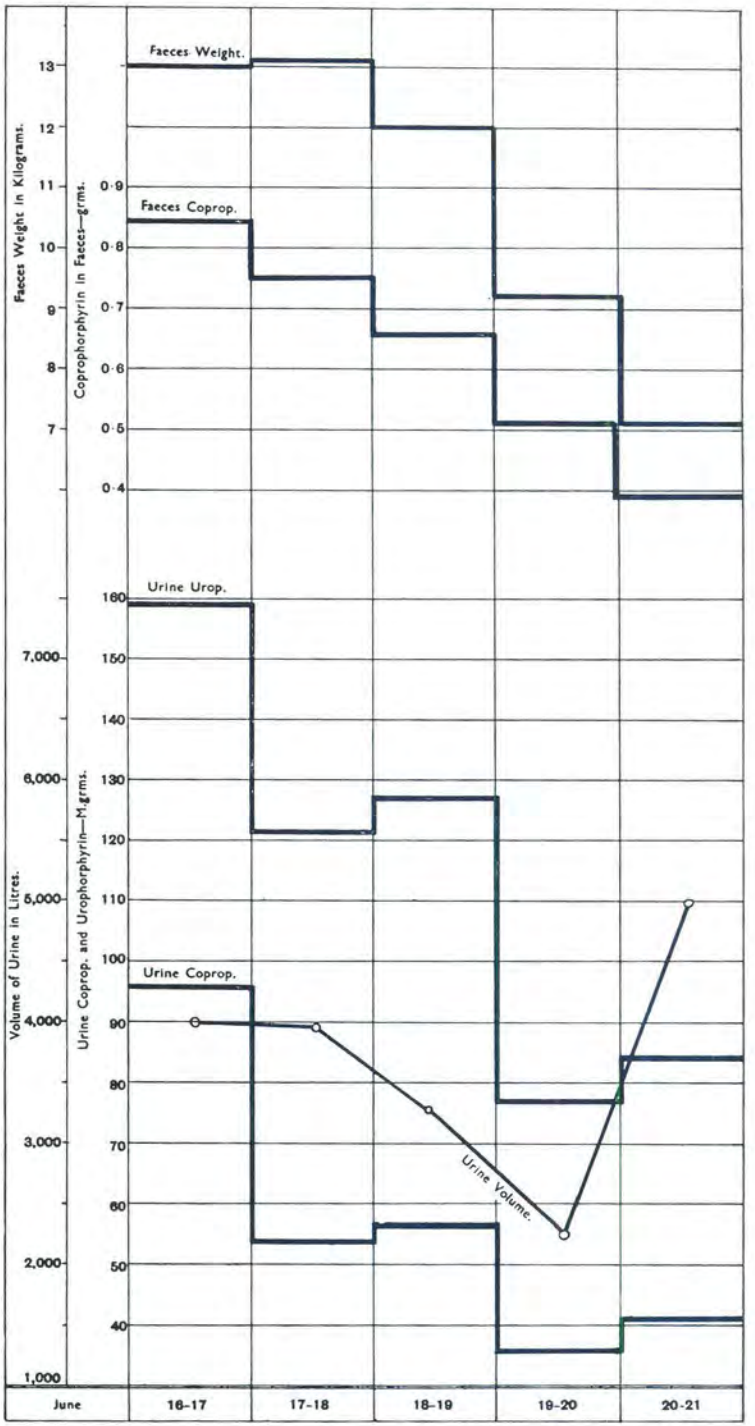
Faeces Weight.
Faeces Coprop.
Urine Urop.
Urine Coprop.
Urine Volume.
Faeces Weight in Kilograms.
Coprophorphyrin in Faeces—grms.
Urine Coprop. and Urophorphyrin—M.grms.
Volume of Urine in Litres.
13
12
11
10
9
8
7
0·9
0·8
0·7
0·6
0·5
0·4
160
150
140
130
120
110
100
90
80
70
60
50
40
7,000
6,000
5,000
4,000
3,000
2,000
1,000
June
16–17
17–18
18–19
19–20
20–21

Fig. 11.

It is clear from an examination of this data that the excretion of porphyrin by any one animal varies greatly from day to day. A similar circumstance has been found in the case of normal dogs by Dobiner (1937c). The period of three consecutive days utilised for the first observation (February 10-12) is clearly too short to allow of any calculation of an average daily excretion. When however the periods March 1-6 and June 16-20 are compared it is sure that both bovine 7017 and 7018 excreted on the daily average considerably more porphyrin during the later period. That this was not due to any error in technique was proved by making up new standards and repeating the observations. We can only suggest that possibly season or climate influenced the porphyrin excretion for it may be noticed that the weather during March was still hot whilst June was cold with temperatures well below freezing point at night. Whether or not this explanation is correct can only be ascertained by an extended series of observations throughout the year.

In assessing the significance of the data presented, the following points should be borne in mind:—

1. Both coproporphyrin and uroporphyrin are of endogenous origin.
2. Porphyrin excretion takes place via the bile and urine thus the relative quantities appearing in the faeces and urine will depend largely upon the balance of factors effecting biliary and renal secretion.
3. Uroporphyrin is also retained in the body being absorbed by growing bony tissue.
4. The exact generic relation between coproporphyrin and uroporphyrin is not known. There is a possibility that uroporphyrin may arise in part from coproporphyrin in the kidney.

An examination of the data does not reveal any precise quantitative relationship between the two types of porphyrin. In seeking correlations between urine volume and porphyrin excretion, the period June 16-20 is the most satisfactory since during this period very wide fluctuations occurred both in urine volume and faeces weight. It would appear that:—

1. There is no correlation between either urinary copro- or uroporphyrin and urine volume. In fact the tendency is towards a constancy of porphyrin output irrespective of the total quantity of liquid passed. Such behaviour is in opposition to that seen in lead poisoning where the quantity of coproporphyrin III voided in the urine rises and falls with the urine volume (Mertens 1937).
2. Coproporphyrin and uroporphyrin seem to run a parallel course in the urine, the quantities of these pigments rising and falling in sympathy.
3. The output of coproporphyrin in the faeces is related to faeces weight. This does not indicate any exogenous origin but is rather an expression of the circumstance that more bile passes into the alimentary tract and is voided when a large bulk of food material is passing through.

4 The total quantity of porphyrin excreted daily is subject to wide variations being chiefly influenced by the magnitude of the faeces component, but remains approximately constant when the daily average over a five or six day period is considered. The highest excretion recorded was 1·27 gm. porphyrin during 24 hours.

5. Further work must be undertaken to ascertain whether or not there is any seasonal rhythm of porphyrin excretion.

Identification of the Porphyrin Excreted.

From the urine of these cases uroporphyrin ester of M.P. 275-7° was isolated and resolved into uroporphyrin I (M.P. 292-3°) and smaller quantities of uroporphyrin III (M.P. 260°). (Rimington 1936.)

In the case of coproporphyrin fraction, this has recently been separated by Rimington and Roets (1937) into coproporphyrin I and coproporphyrin III. It was highly desirable, however, that the relative proportions of these two isomers should be known and accordingly the following experiment was performed with this object in view:

2·8 litres of freshly passed urine (bovine 7017) was acidified by acetic acid and extracted in a Kutscher-Steudel apparatus with ether until no more porphyrin was removed by the solvent. The ether solution was then worked up in the usual way and the total quantity of coproporphyrin measured by comparison of the 5 per cent. acid solution with the standard coproporphyrin.

Volume of solution 500 c.c.

5 c.c. aliquot was diluted to 29·5 c.c. to match standard of 1 mgm./100 c.c. therefore 29·5 mgm. porphyrin in all.

The pigment was transferred to ether, esterified and the total ester obtained by evaporating the chloroform solution to dryness. On stirring with cold methyl alcohol, a portion dissolved. This was coproporphyrin III and its quantity was ascertained by matching against the standard. The quantity of coproporphyrin I remaining was similarly determined.

Coproporphyrin	III	1·06 mgm.
,,	I	29·11 mgm.

In order to be sure that No. I series isomer accompanied the III, the methyl alcoholic solution of the latter was evaporated to dryness and again treated with cold methyl alcohol when it dissolved completely. The M.P. of the crystalline material was 120-130°. The purified coproporphyrin I fraction had M.P. 245-7°. From the above figures it is seen that in the urine of this animal the

ratio $\frac{\text{coproporphyrin I}}{\text{coproporphyrin III}} = \frac{27{\cdot}6}{1}$

Similar experiments are in progress to determine the ratio in the remaining cases and also the ratio exhibited by the faeces porphyrins.

Repeated recrystallization of a coproporphyrin I specimen isolated from faeces had M.P. 250°.

REFERENCES.

DOBINER, K. (1936). Simultaneous excretion of coproporphyrins I and III. *Proc. Soc. Exp. Biol. Med.*, Vol. 35, pp. 175-176.

DOBINER, K. AND BARKER, W. (1937*a*). Total coproporphyrin I excretion in pernicious anaemia. *Proc. Soc. Expt. Biol. Med.*, Vol. 36, pp. 864-867.

DOBINER, K., STRAIN, W., LOCALIO, S., KEUTMANN, H., AND STEPHENS, D. (1937*b*). Coproporphyrin I metabolism and haematopoietic activity. *Proc. Soc. Expt. Biol. Med.*, Vol. 30, pp. 755-756.

DOBINER, K. (1937*c*). The excretion of porphyrin by dogs. *Proc. Soc. Expt. Biol. Med.*, Vol. 36, pp. 757-761.

FINK, H. AND HOERBURGER, W. (1935). A further case of ochronosis in slaughter animals. *Zeit. Physiol. Chem.*, Vol. 232. pp .77-78.

FISCHER, H. AND HOFMANN H. (1937). Constitution of uroporphyrin and mussel porphyrin. *Zeit. Physiol. Chem.*, Vol. 246, p. 15.

FOURIE, P. J. J. (1936). The occurrence of congenital porphyrinuria (Pink tooth) in cattle in South Africa (Swaziland). *Onderstepoort Jnl. of Vet. Sci. and Animal Industry*, Vol. No. 2, pp. 535-566.

FOURIE, P. J. J. AND RIMINGTON, C. (1937). *Nature*, Vol. 140, No. 3532, p. 68.

GARROD, A. (1923). Inborn errors of metabolism. 2nd Ed. London.

GÜNTHER, H. (1911). *Deut. Archiv. f. Klin. Med.* No. 89.

GÜNTHER, H. (1922). *Ergeb. d. Allgem. Path. u Path. Anat.* (Lubarsch, Ostertag), Vol. 20 No. 1, p. 643.

HEGT AND HAUSMANN (1920). *Stralen therapie*, Vol. II, pp. 444.

MERTENS E. (1936). The uroporphyrin of acute haematoporphyria. *Zeit. Physiol. Chem.*, Vol. 238, pp. 1-11.

MERTENS, E. (1937). Excretion of coprophyrin III in lead poisoning. *Klin. Woch.*, Vol. 16, pp. 61-62.

RIMINGTON, C. (1936). Some cases of congenital porphyrinuria in cattle: Chemical studies upon the living animals and post mortem material. *Onderstepoort Jnl. of Vet. Sci. and Animal Industry*, Vol. 7, No. 2, pp. 567-609.

RIMINGTON AND ROETS. (1937). *Nature*, Vol. 140, No. 3544, p. 584.

RIMINGTON C. (1937). *Nature*, Vol. 140, No. 3533, p. 105.

VAN DEN BERGH, H., REGNIERS AND MULLER, P. (1928). *Arch. f. Verdanungskheiten*, Vol. 42, p. 306.

VAN DEN BERGH, H. AND GROTEPASS, W. (1937). A notable case of porphyry. *Wiener. Klin. Woch.* 1937, No. 21.

WALDENSTRÖM (1935). *Deut, Archiv. Klin. Med.*, Vol. 178, pp. 38-49.

APPENDIX.

Bovine No. 7017.

Date.	Faeces.		Urine.			Total porphyrin gm.
	Weight Kgm.	Coprop. mgm.	Volume litres.	Coprop. mgm.	Urop. mgm.	
February—						
10	9·90	545·0	2·93	33·2	64·5	0·6427
11	8·70	870·0	2·89	62·6	74·2	1·0068
12	9·00	675·0	3·38	49·1	128·9	8·8530
March—						
1	6·20	418·6	2·82	74·1	64·0	0·5567
2	10·90	626·9	2·10	57·8	59·8	0·7444
3	11·60	664·1	3·71	71·9	64·0	0·8000
4	10·10	636·3	3·73	65·3	67·1	0·7687
5	12·50	551·4	4·50	64·7	87·7	0·7038
6	9·30	*627·8*	2·76	*60·4*	*66·2*	0·7544
Mean	—	587·5	—	65·7	68·1	0·7213
June—						
16	6·90	690·0	3·77	90·4	150·6	0·9310
17	9·2	1,012·0	3·28	91·8	166·2	1·2700
18	6·8	884·0	5·30	84·8	155·5	1·1243
19	5·7	627·0	2·95	70·8	118·0	0·8158
20	4·7	*564·0*	7·20	*70·1*	*131·0*	*0·7651*
Mean	—	755·4	—	81·6	144·3	0·9812

Bovine No. 7018.

Date.	Faeces.		Urine.			Total porphyrin gm.
	Weight Kgm.	Coprop. mgm.	Volume litres.	Urop. mgm.	Coprop. mgm.	
February—						
10	12·40	477·0	3·17	56·5	74·3	0·6078
11	12·90	537·0	2·05	39·7	146·5	0·7232
12	11·50	409·0	1·88	20·9	73·5	0·5034
March—						
1	13·90	469·2	2·12	23·6	48·1	0·5409
2	10·50	420·0	1·44	23·0	46·1	0·4891
3	13·10	467·9	2·34	21·1	48·4	0·5374
4	13·00	435·2	3·19	23·4	45·7	0·5043
5	10·5	525·0	6·90	50·6	42·1	0·6177
6	8·7	*398·7*	4·90	*17·2*	*71·9*	0·4878
Mean	—	452·7	—	26·5	50·4	0·5295
June—						
16	12·95	841·8	4·00	96·0	159·4	1·0972
17	13·10	786·0	3·95	54·1	121·7	0·9618
18	12·00	660·0	3·29	56·4	126·9	0·8433
19	9·20	510·6	2·25	35·9	77·0*	0·6235
20	7·10	*390·5*	4·95	*41·1*	*84·0**	*0·5156*
Mean	—	637·8	—	56·7	113·8	0·8083

* Possibly some loss by overflowing.