Copper metabolism in milch cows. III. Evaluation of the method for radio-measurements over the liver

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Summary

The method for external liver measurement of ^{64}Cu in cows has been further evaluated. In vivo and in vitro experiments are described from which the absolute and relative error of the measurements are calculated. The percentage uptake on the average had 2.1–2.3 % standard deviation. The error in biological half life was found to vary from 4.5 up to 65 %, depending on the length of the observation period. Conditions are specified for optimal performance of the method. This is considered quite satisfactory for a number of explicitly described types of experimentation.

1. Introduction

In the first of the preceding communications a general outline has been given for radioactive measurement in vivo of copper in liver. Despite the short physical half life of the isotope ^{64}Cu the graphs for liver reading vs. time showed well-defined uptake and discharge lines. From these lines the maximal uptake and the biological half life could be calculated without difficulty.

Once this has been demonstrated the prerequisites for the use of the method and its technical performance must be considered critically. The following subjects will be treated here: the geometry of the body related to the counting system, the position of the counter, its shielding and the value of background readings, the relative accuracy of the determination and its consequence for the estimation of uptake and half life values.

2. The geometry of the body related to the counting system

The liver itself should be the first subject of investigation. It is very far from the ideal point source. This means that even at 20 cm from the body the contribution to the total count rate will be unequal for the radio copper in various liver parts.

The second item for study is the contribution to the count rate from radio copper in other body compartments. This contribution is as yet ill-defined.

2.1. The liver topography and geometry

The starting point of these considerations is that in cows the liver copper is fairly

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Table 1. Total copper and radioactivity in different parts of cow's liver

Part of liver (organ)	*Total Cu (I)*	*Radioactivity*			*Radio copper (I)*[2]
		(I)[1]	*(II)*	*(III)*	
Lobus caudatus	268 ± 3[3]	330 ± 70[4]			0.032 ± 0.004
Lobus dexter med.	263 ± 5	—		210	—
Lobus sinister lat.	283 ± 7	315 ± 70	460		0.027 ± 0.003
Gall bladder			360	160	
Right kidney		≪ 50 ± 70			

[1] (I) cow slaughtered 72 h post injection (intramuscular), (II) and (III) after ± 42 h post intravenous injection. Measurement of whole lobe or organ in total body counting position. Result expressed as count rate per minute corrected for background.

[2] The same cow (I). A homogenate of whole lobe was made and 4 ml counted in a well counter. Result expressed as % dose ± standard error of single determination.

[3] In mg per kg d.m. with standard deviation of the average of 6 determinations in two samples of each lobe.

[4] The standard error is high as consequence to low count rate (total about double background).

homogeneously distributed (e.g. VAN DER GRIFT, 1955). In a limited number of experiments this could be confirmed not only for the total copper content but also for the radio copper, administered intramuscularly (Table 1).

BOWLAND et al. (1961) and CASSIDY and EVA (1958), in studies with pigs, have found relatively large variation in total copper content. It should be remarked, however, that the *average* content in the different lobes, also in their results, did not fluctuate appreciably. Variations of copper contents in liver tissue on a micro scale have also been revealed in histological investigations (WACHSTEIN, 1963). Such variation in radio copper, upon integration at sufficient observation distance, will be much less important, however, than variation between lobes. From the results in Table 1 plus the experience described in literature it seems legitimate to conclude that any deviation caused by unequal distribution of radioactivity will be small compared to the inherent error of the determination.

The remaining difficulties then are mainly consequent to two factors: the large liver size and the aspherical, lobular shape. Also the location of the liver can sometimes be important. We will consider these factors in individual animals through a series of experiments and then in inter-animal comparisons.

It is known that the liver mass in an individual during different treatments is not always constant. For instance higher needs for detoxification or higher general activity will bring about enlargement. From the results in Table 2 it will be seen that stall- or pasture feeding will not necessarily influence the results of radio-measurement. In this table the ratio's are recorded between the count rates at the skin and those at the usual 20 cm distance. These ratio's are sensitive to changes in the above mentioned factors (see also the first paper of this series (BINNERTS, 1964).

Normal ratio's seem to be from 3.0 to 3.3. The lower values in the September experiment were obtained with highly pregnant cows in advanced pregnancy. The 10–15% effect stems mainly from the reduction of the skin-readings. As a consequence the normal observations are affected to much less than half the deviation, that is ≪ 5–8%. In this pregnant animals dislocation of the liver was found; the maximum of the readings on the skin was ½ an intercostal distance less caudal than normal, but this effect was not noticed with the usual 20 cm extension. Also remarked in normal animals was an effect by feed intake, probably resulting from compression of the liver in the direction of the counter. During feeding the results directly over the skin became progressively higher, but the 20 cm readings remained unaffected. In conclusion it seems justified to consider the influence by liver geometry changes for the individual animal in not too extreme cases as negligible.

For comparison between animals the difficulties strictly cannot be resolved without consideration of the special anatomy of each cow. It seems, however, reasonable to assume that tentative comparisons may be made of normal animals of the same breed and about equal weight. Still more safe would be to compare the averages of not too small groups of such animals; this will be done in subsequent studies.

For calculation in absolute measurements of the accumulated liver dose, the geometry has been evaluated in different ways. On two occasions the entire liver, including the gall bladder was obtained from cows killed a short time after dosing, while the usual in vivo measurements had already been performed. Also liver phantoms were measured. As a representative example the following calculation is presented.

The in vivo liver measurement showed accumulation to 16.5% relative to the count of a radio copper solution in a 100 ml polythene bottle at 20 cm counting distance. Extrapolation from previous measurements yielded 18.3% at the time of slaughter, 6 h later. The liver, cut into pieces and transferred to a 10 l jar gave count rates equal to 37.8; 38.0 and 36.6% of the dose, average 37.4%. These results are from independant readings, each time performed with the liver pieces anew introduced into the jar to a flat horizontal surface; the standard was diluted to the identical volume. From these in vivo and in vitro results a correction factor for the in vivo

Table 2. Measurement without and with 20 cm extension tube

Name of cow	*Date and stalls (S) or pasture (P)*	*Results (in arbitrary units)*		*Ratio a/b*
		on skin (a)	*with tube (b)*	
Zwartsch. 9	Jan. 1964 (S)	183	56.9	3.22 [1]
	Nov. 1964 (S)	53.5	15.7	3.41
Zwartsch. 11	Dec. 1963 (S)	457	139	3.28
	Nov. 1964 (S)	49.3	14.8	3.33
	Aug. 1965 (S)	17.0	5.2	3.27
	Sept. 1965 (P)	38.6	11.7	3.30
Zwartsch. 12	Aug. 1965 (P)	15.2	4.5	3.38
	Sept. 1965 (S)	46.2	14.8	3.12
Witsch. 10	Aug. 1965 (S)	20.2	6.2	3.26
	Sept. 1965 (P)	21.1	7.4	2.85 [2]
Anna 10	Aug. 1965 (P)	13.1	4.4	2.98
	Sept. 1965 (S)	20.0	7.2	2.78 [2]
Aaltje 21	Nov. 1963 (P)	10.1	4.7	2.15 [3]
Aaltje 22	Nov. 1963 (P)	7.1	3.3	2.15 [3]
Lamkje 9	Nov. 1964 (S)	34.5	12.3	2.80 [4]

[1] The standard error of the average is calculated ± 0.10.
[2] This is one month before calving (however note the preceding values at 2 months).
[3] Animals from a different farm, four months before calving.
[4] Very fat cow, three months before calving.

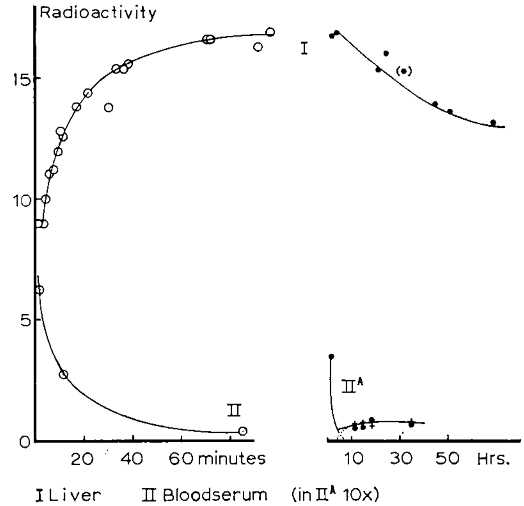

Fig. 1. Radioactivity over the liver and in blood samples after intravenous administration of radio copper.

results is obtained of 37.4/18.3 = 2.04. This result is very near to the general average of the other experimental results, though the phantom considered best gave a result somewhat higher than 1.90. It is felt that constructing liver phantoms is very difficult, especially so because some shrinking may occur after slaughter by bleeding, while in the dissection much of the original shape is lost. The most satisfactory method for conserving the original volume and shape of the liver would therefore be freezing after thorough clamping of the blood vessels. However, no further activity was developed in this direction because it was realised that individual variation would exclude precision measurements in practice and also that no precision better than a few per cent can be obtained in the routine experiments in the individual animal (compare section 4).
Consequently, as a general procedure in routine measurements, the liver results obtained in vivo and compared to the standard dose in 100 ml polythene bottles at 20 cm counting distance, are corrected to absolute value by multiplication by the factor 2.0. In this way all the reported results have been obtained.

2.2. The contribution of other body copper

Upon *intravenous* injection the initial counts originate probably from other body copper, in this case from the blood. In Fig. 1 the effect is seen in the very first minute after the injection had been completed (it had been ascertained before that no appreciable contribution was given by the fluid in the syringe at the injection site). But after some time, always within a few hours, the blood radioactivity has declined to insignificant levels.
Immediately after *intramuscular* injection no counts are registered over the liver. It may be remarked that although the scintillator crystal is not collimated, it has the normal cylindrical shielding of 2.5 cm of lead and further that a thin 0.15 cm lead filter is surmounted. These precautions will prevent most of the tangential primary and virtually all secondary radiation from entering the crystal. Moreover it should

be realised that the distance from the injection site (in the neck or in the gluteal muscles) is relatively large compared to that in smaller experimental animals.
With intravenous injection the maximum over the liver is recorded well within 24 h; with intramuscular injection it usually takes somewhat more time; in our experience up to three days. In both ways uptakes between 20 and 70% have been recorded, with on the average about 50% (35% in pregnancy, see next paper). Thus an average 50% or more of the dose is deposited elsewhere in the body, a small part of which will be excreted within the next few days. Typical patterns of secretions and excretions are reproduced in Fig. 2. It will be readily seen from this figure that the sum of secretions and excretions after a couple of days is determined solely by the fecal loss, in these instances 0.5% daily or less compared to the initial dose [1].

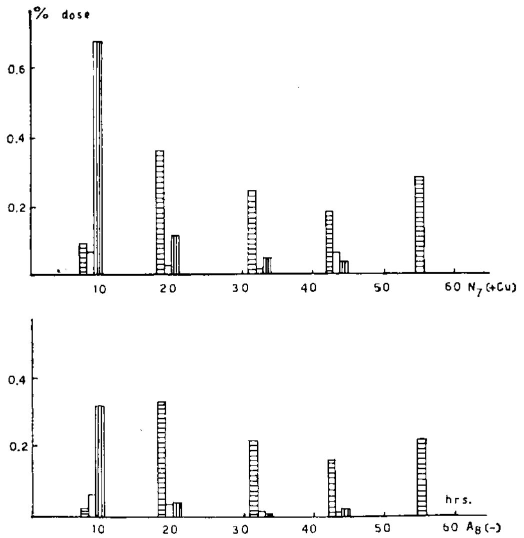

Fig. 2. Secretion and excretion of radio copper after intravenous administration. The columns, from left to right, represent total quantities in percentage of dose in feces, urine and milk, respectively. The upper graph for the cow Nellie 7 was obtained with oral administration of 1 g of copper-sulphate two times daily in gelatine capsules. The other cow, Annie 8, did not receive additional copper. The relatively high secretions in the first milking are remarkable.

From the copper outside the liver some will be found in specific organs and tissues. It is known for instance that the kidney and the spleen have quite important copper contents (UNDERWOOD, 1953) and thus, as well as the pancreas, would seem to contribute appreciably to the radiation in the liver area. It is fortunate, however, that the radio copper does not reach its final equilibrium during the experimental period. It is found experimentally that the radiation from e.g. the kidney remains insignifi-

[1] It should be remembered that this does not represent the body copper lost in toto, since equilibrium is not yet attained. If the recorded 0.5% loss is thought to be derived entirely from the liver then the half life of the radio copper in the liver would be 5000 h. The much shorter values observed point to important secretion from the liver into the body. Reabsorption from bile and subsequent redistribution of radio copper will also cause (limited) interference.

cant, even after 4 days of the experiment (Table 1). This is in accordance with a result published by COMAR (1950).
The radio copper, apart from these specific centres, is diffusely distributed within the body and this will cause only limited interference because of the enormous body mass of the mature cow and the large average distance to the counter (with, again, most of the tangential component of the radiation absorbed by lead). Table 3 gives some results of experiments in which a 70% dose was simulated in a large volume, resembling the body of a cow and 30% in a confinement similar to that of the liver. It appears that the interaction with the liver counting would amount to 140 in a total of 1290, that is less than 11%. An observed liver uptake of 33% would therefore be in reality 30%. With the normally observed 50% uptake (see following paper) the interaction would be only 5/7 × 140 counts on areal liver count 5/3 × 1240, or less than 5%. The observed liver dose would then be 52% in stead of 50%. These are small effects indeed, and in practice the interferences will be still smaller because of the favorable geometry of the bovine body, having part of the large stomach masses under the liver in the field of "vision" by the crystal. These masses after injection have only small amounts of radioactivity (e.g. totalling about 1% of the dose, COMAR (1950). (Discussion of *oral* administration will be reserved to a later opportunity.)

Table 3. The influence on count rate by the dispersed radio copper

Radioactive model	*Count rate per minute*
30 % dose in ± 4 l	1240 ± 40 [1]
Idem + 70 % dose in ± 300 l [2]	1290 ± 40
70 % dose in ± 300 l	180
Idem, corrected	140 [3] ± 15

[1] Standard deviation of the average.
[2] Two large vessels of 116 × 51 × 25 cm directly above each other and filled with water. The flat smaller 4 l volume was placed in contact with the wall in the upper vessel and the counter directed at this volume at 20 cm at some angle from the exterior. The mixing in the large vessels was controlled with fluoresceïne.
[3] Corrected for radioactivity at the site of the removed 30 % dose.

3. The counting system

Some reasoning on the counting system related to the geometry of the liver has been given in the first of these communications. Therefore only a few remarks will be made. Further discussion is needed on the shielding of the crystal and on the value of background readings, especially so because these may influence strongly the low results towards the end of the experimental periods.

3.1. The position of the counter relative to the body

The counting system should give results unaffected by small variation in distance and in location. The reading of counts therefore should be best practised with the 20 cm extension tube. Nevertheless, also direct reading over the skin is performed

routinely when the amount of radioactivity has become small. Special care is given to the performance of the readings then, by observing during longer periods. They are executed 5 in stead of 3 times and averaged.
Theoretically these results should be compared to that with a more sophisticated type of liver phantom, which would cause grave difficulties. In practice such devices are seldom needed. Before the radioactivity has decreased to such low values that skin contact is needed, liver readings are simultaneously performed with and without extension tube and they are compared. Thus a factor of multiplication is derived, which can be used to transform the subsequent results to the normal level. It is quite well possible then to extend the period of observations to more than one week, even with a limited dosage of one millicurie or less. But injections with doses up to 10 mC cause little difficulty neither by radiation nor by the induced inorganic copper load and they also permit sufficiently long measuring periods.
The maximal activity with and without extension tube is usually discerned at the 11th rib. The counter tube or its extension is then perpendicular to the body surface and pointing slightly downward. Displacement of up to 1 inch in any direction usually will not cause significant aberration.

3.2. The value of blank readings

In the measurement of very small quantities of radioactivity considerable influence can be exerted by the animal's body mass. This is not primarily by its radioactivity but on the contrary by shielding of a considerable part of the lower energy background radiation. In Table 4 some results are given of readings of blanks obtained

Table 4. The background in the air compared with the background over untreated cows [1]

Without filter		*With 1.5 mm lead filter*	
air reading	*untreated cow*	*air reading*	*untreated cow*
720	540	350	380
730	550	350	330
700	580	400	390
730			
750			

[1] Scintillation counter connected to count rate meter. Results expressed in count per minute. Equilibrium time ½ minute at final condenser capacity setting.

by placing the counter away from animals in the open, compared to blanks measured over untreated cows. (This influence was not recognized in the first experiment with oral dosage, which resulted in the steep lines for liver copper discharge recorded in Fig. 2 of the first paper (BINNERTS, 1964). With correct appreciation of the zero counts the lines are less steep and resemble much more the normal finding after administration by way of injection.)
The above mentioned effect will not have influence to any appreciable extent on the results after injection, because the counting was sufficiently high then to permit the use of a 1.5 mm lead filter. This while absorbing virtually all of the low-energy

radiation, absorbs only an average 20 % of the 0.5 MeV positron disintegration radiation of the ^{64}Cu. (The normal aluminium screw cap directly over the crystal will absorb any scattered electrons from the lead filter.) The fact that the background is not reduced to zero is caused mainly by the characteristics of the multiplyer tube ("noise").

From Table 4 its follows that with the filter in position no difference can be found between the air and cow background readings. This means that liver measurement starting from $\pm$ 72 h onward seems also possible after *oral* dosing. Thus the possibilities of the method will be extended appreciably, although it should be remembered that much higher doses are needed because of the low and slower resorption. Where doses of 0.5 (real) mC, preferably 2.5 mC, are sufficient for the injection method, oral administration will take at least 20 mC per caput with the present apparatus. With the present method of preparation of the radio copper this would require a gross 50 mg of Cu. Such a quantity would not be unphysiological for oral intake in cows. Undoubtedly, much lower doses could be used with whole body counters, but then the total amount of copper absorbed will be measured and not specifically the liver dose. This of course will give a good tool in resorption studies.

4. The relative error of the determination

With the precautions described a normal set of three (at low radioactivities of five) independant readings over the liver is carried out and the results are averaged. In the following statistical treatment the thus obtained averages will be termed "observations".

In this statistical treatment the extremely low activities will not be considered; a couple of times the blank reading is maintained as a minimum. The problem, what statistical weight to ascribe to the other results in different activity regions, was circumvented by the realisation that with the lower activities higher sensitivity on the count rate meter and longer equilibrium time had been applied than with the higher activities. This will counteract any extra error caused by the larger correction for physical decay of the later readings. Indeed calculation for a limited number of cows did not reveal systematic differences in the obtained relative error between the readings at 40 and these at 100 h experimental time. For the computation of the relative error in all instances the deviation was calculated from the ideal straight line fitting the points of observation in the semilogarithmical plot.

In 31 of the observations obtained with 4 cows, the deviation was a mean 4.40 %, against only 2.68 % in 13 determinations with standard solutions in 100 ml bottles under otherwise identical conditions. The results differed as expected between the different cows. In one cow the result 2.32 % (9) was even a little lower than in the standards, in another cow it was a high 8.55 % (7), in a third and fourth cow 4.20 % (8) and 4.58 % (7), respectively (the number of observations is recorded in parentheses).

In a larger body of results the standard error of the determination in cows was 5.2 % (106 values of 18 injection experiments with 14 different cows considered). Thus the obtained maximal liver uptake value (U) will have a standard deviation of 5.2 $\sqrt{N}$ %, in which the number of observations, N, usually is 5 or 6, so that the standard error in U will then be 2.1 %–2.3 %. The standard error of the biological half life ($t_{1/2}$) can be computed as: $t_{1/2} / \triangle t \,.\, \triangle U$, in which $\triangle t$ is the length of the total observation period. In the normal procedure the observation times are approximated to whole hours, but because of the small slopes of the obtained straight lines, this will not appreciably influence the standard deviation. In the recorded experiments with intramuscular injection, owing to the time lag for absorption, the period during which the semilogarithmical relation was fulfilled on the average was limited to 72 h, which is small compared to the biological half life of the liver copper. The standard error of the determinations of half life ran therefore much higher than that of the uptake; from $140/72 \times 2.3\% = 4.5\%$ up to $2000/72 \times 2.3\% = 64\%$. With intravenous injection a somewhat longer observation time can be applied, but never much over 100 h; thus the deviation of the half life determination remains large.

5. Discussion

In order to improve the absolute and relative accuracy of the method several precautions have been taken. From the sections 2 and 3 it is clear that the counter should be placed on a fixed place, preferably the 11th rib, at the location of maximum count rate. Then the counter should best be provided with a filter (1.5 mm of lead), not further collimated than with the normal lead cylinder around the crystal and placed at the fixed distance (20 cm) from the skin. In this way and when the measurement is confined to milk cows of 450–600 kg, the absolute error will not be large. It is realised, however, that comparing results within a group of animals of different size is very hazardous, unless sufficient accurate liver phantoms can be constructed. Experiments of comparison should therefore be well devised with respect to the size of the animal. Such comparisons promise to be even quite satisfactory with smaller groups of such animals. Regarding the relative error much could be gained by applying longer observation periods. This is possible by using higher doses, applying intravenous injection or eventually, if this is possible, by speeding up the absorption after intramuscular injection. For the same reason subcutaneous administration is to be condemned. It should be remembered, however, that the intravenous injection should be given under special precaution, so that no risk of colloid formation exists and the load of total copper should not be unphysiologically large. The use of complexes, like that of glycine, slowly administered by a previously inserted nylon tube into the vein, may be the most satisfactory. The frequency of observations cannot be easily augmented because of interference with the normal life of the animals. By careful consideration of the peculiarities of the copper distribution and of the conditions for measurement a reliable estimation seems possible of radio copper in liver. The absolute error of the determination can be kept satisfactorily small and the relative error of the calculated "uptake" values is slightly over 2%. The half life of the liver copper will not be estimated very accurately, but semiquantitative estimations seem very useful. The method seems suitable for practical application.

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