THE COPPER METABOLISM OF DROSOPHILA*

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The copper metabolism of insects has been discussed in the past by various authors, mainly on the basis of analyses of whole animals. In the fly *Lucilia cuprina*, Waterhouse[1] studied by sensitive histochemical methods the uptake of copper and its distribution to the tissues. The histochemical findings were supplemented and supported by careful chemical analytical methods.

One of us has made, and in part reported elsewhere[2-4] similar histochemical studies on a number of species of *Drosophila*. However, the work of Okamoto *et al.*,[5] on copper in tissues and our knowledge of such copper proteins as hemocyanin[6] and ascorbic acid oxidase[7] indicate the presence in tissues of copper compounds which will not react in the various histochemical tests. It thus seemed desirable to extend our observations by tracing the copper metabolism of *Drosophila* larvae with the radioisotope, Cu^{64}. This has the advantage of providing both a somewhat more sensitive test for copper and one which is independent of the state of combination of the copper in the tissues.

Methods.—The radioisotope Cu^{64} was produced[8] by neutron irradiation. The C. P. metal was cleaned in advance and irradiated wrapped in cleaned aluminum foil. After short (36–48 hrs.) periods of irradiation, careful determination of decay curves of various samples has confirmed that no significant contamination by other radioisotopes has been produced. Accordingly no chemical separation of the irradiated copper was made.

The irradiated copper, carefully weighed before irradiation, was dissolved in HNO_3-H_2SO_4 and the HNO_3 driven off by heating to SO_3 fumes. The solution was made up to contain a known weight of copper per gram, and suitable aliquots were added to Drosophila medium[9] or to yeast media. By mixing the Cu^{64} solution into 50–100 g. of hot fly food, high uniformity of distribution was secured as measured either radiochemically or by chemical analysis.

Detection of radioactivity has been by means of conventional Geiger-Müller counting of whole or dissected larvae; of contact auto-radiography of dissections of larvae; and of histological autoradiography of paraffin sections. Data obtained by the two latter methods will be presented here only in so far as it relates to (confirms or extends) the counting observations, and will be reported in full elsewhere.

By parallel counts on aliquots of the original Cu^{64} solution, and on weighed portions of the radioactive food, and by giving due attention to decay cor-

rections and to uniformities of counter response, it has been possible to convert much of the data from counts per minute directly to micrograms of the original copper sample. Although the energies of Cu^{64} β particles are low (0.57 and 0.66 Mev max.), the very small mass of the tissues being counted has permitted us to neglect self-absorption corrections, and to obtain results of adequate precision.

The larvae used were raised from eggs collected over 12-hour periods. Early third instar larvae selected for uniformity of size were used in all the experiments. Except during the time required for manipulation and transfer the larvae were kept in an incubator maintained at 25° ± 0.1C.

Larvae were scoured of radioactive food by transfer for periods of 2 hours or more to non-radioactive medium. Before preparation for counting or

TABLE 1

UPTAKE OF CU BY DROSOPHILA LARVAE. EFFECT OF CANDIDA ALBICANS (TEXAS Y 12)

SPECIES	Cu^{64} LEVEL, μG./G.	TIME ON	TIME OFF	YEAST	C./MIN./ LARVA
Repleta	2.5	8	4 1/2	+	108
	2.5	8	3	−	113
	2.5	8	5 Cu	−	101
	0.25	8	4	+	10
	0.25	8	3 3/4	−	11
Melanogaster	2.5	8	6 3/4	+	28
	2.5	8	4 3/4	−	35
	0.25	8	7	+	6
	0.25	8	6 1/4	−	2
Ananassae	2.5	8	7 1/2	+	36
	2.5	8	5 1/2	−	43
	0.25	8	7 1/4	+	4
	0.25	8	6 1/2	−	7

NOTE: Similar data obtained for *Saccharomyces* (baker's yeast).

fixation larvae were thoroughly washed in *Drosophila* Ringer solution. Larvae used for whole counts and sectioning were fixed in hot formalin-alcohol. For contact autography larvae were dissected without fixation on dry slides on which the organs were spread and subsequently oven-dried at 60° for 30 minutes. For counts of dissected larvae the dissection was performed in the dishes in which counting was to be carried out and parts transferred to other dishes so that there was no loss of material or activity.

Experiments and Results.—Since a major fraction of the food of *Drosophila* larvae consists of the yeast cells growing on the medium, it was first desirable to examine the effect of yeast on uptake by larvae of copper from the medium. The data from one experiment designed to test this are set out in table 1. The copper was mixed into the hot fly

food, in the concentrations indicated and replicate samples were taken. One of each pair was inoculated with yeast which was then allowed to grow for 12 hours. The other was left sterile. Larvae were placed on the two types of media for 8 hours and removed as indicated in the table. The uniformity of counting rate of larvae taken from the same copper concentrations indicates that even though there had been very appreciable yeast growth on the inoculated cultures, the yeast cells have no effect on the amount of copper absorbed by the larvae. Similar experiments over other and longer times and using *Saccharomyces* as well as *Candida*, confirmed this.

The effect of varying concentrations of copper in the medium on the amount taken up by the larvae at constant time, is illustrated in table 2. Although uptake ceases to be strictly proportional to concentration for *ananassae* between 10 and 25μg. Cu per gram of medium, and for *repleta* between 2.5 and 5μg. Cu per gram, the control of uptake is not strict. In

TABLE 2

UPTAKE OF Cu^{64} BY DROSOPHILA LARVAE. EFFECT OF TOTAL CU IN THE MEDIUM

TOTAL CU, μG./G.	CU CONTENT AFTER 8 HRS. ON AND 4–6 HRS. OFF, DIVIDED BY THAT AT 2.5 μG. CU/G.		
	REPLETA	MELANOGASTER	ANANASSAE
0.25	0.1	0.1	0.1
2.5	1	1	1
5.0	1.4	...	2
10.0	2.6	...	4
25.0	5.2	...	5.6
100	13.4	24.6	21.4
200	21.5	13.3	15.1

repleta even at 200 μg. Cu per gram, there is no evidence of a real plateau in the curve relating uptake to concentration. With the smaller *ananassae* and *melanogaster*, increase has ceased and an actual decrease in uptake is observed between 100 μg. Cu per gram and 200 μg. per gram. Typical symptoms of copper toxicity make themselves evident at higher concentrations.

A similar difference in capacity for copper uptake is seen in table 3 which illustrates the relation between copper content and time on the medium for *repleta* and *ananassae*. In these experiments larvae were allowed to feed on radioactive food for periods of 4 hours, 8 hours, 16 hours and 24 hours respectively, and then on non-radioactive food for 2 hours before preparation for counting. It is clear that uptake at the several concentrations reaches a plateau after about 16 hours on the Cu^{64} medium in both species. Accordingly 16 hours has been used as the feeding time in the experiments on excretion.

Excretion of copper has been followed by determination of activities at

intervals after the standard time of 16 hours on food containing Cu^{64} at concentrations ranging from 2.5 to 25.0 μg. Cu/g. A typical experiment on the excretion of absorbed copper is illustrated in table 4. Here two hours has been taken as the standard flushing out time, since it is established that the solid contents of the gut are wholly passed through in somewhat less than 2 hours. In the case of copper we do not seem to have the problem of unabsorbed material lying for long periods between the peritrophic membrane and the gut epithelium as has been described for barium[10]. Although in this experiment the *ananassae* gave very variable results, it is plain that both species do excrete copper absorbed from the medium. In *repleta* excretion proceeds more gradually and steadily to a lower level of retention

TABLE 3

UPTAKE OF Cu BY DROSOPHILA LARVAE

EFFECT OF TIME ON FOOD, $\frac{\text{C./MIN./LARVA AFTER T ON AND 2 OFF}}{\text{C./MIN./LARVA AFTER 4 HRS. ON, 2 OFF}}$

TOTAL CU,	REPLETA				ANANASSAE			
μG./G.	4 HRS.	8 HRS.	16 HRS.	24 HRS.	4 HRS.	8 HRS.	16 HRS.	24 HRS.
2.5	1	2.4 2.9	5 4	5	1	1.6 1.0	8 5	9
5.0	1	2.5	6.5	6.1	1	4	5.7	5.7
10.0	1	2.5 2.4	10	10	1	2	10	3.5
25.0	1	2.25	4.5	5	1	1	2.2 2.0	3.2

TABLE 4

LOSS OF CU^{64} ON PLAIN FOOD: C./MIN./LARVA (16 HRS. ON CU^{64}) RATIO $\frac{\text{AT T OFF}}{\text{AT 2 HRS. OFF}}$

TOTAL CU,	REPLETA				ANANASSAE			
μG./G.	2 HRS.	4 HRS.	8 HRS.	16 HRS.	2 HRS.	4 HRS.	8 HRS.	16 HRS.
2.5	1	0.68	0.55	0.25	1	0.65	0.57	0.80
5.0	1	0.67	0.57	0.30	1	0.93	0.83	1.43
10.0	1	0.29	0.39	0.20	1	0.48	0.36	0.42
25.0	1	0.76	0.45	0.30	1	0.92	1.2	0.68

while such copper as is excreted by *ananassae* is lost quite quickly and the fraction retained is rather higher. The significance of this will be discussed further below.

Although a variety of experiments were tried using food enriched with different amounts of stable copper for flushing out, no evidence was obtained for an increased rate of excretion under these conditions. This is illustrated in lines 2 and 3 of table 1. The line marked "Cu" represents larvae scoured on food containing 25 μg. Cu per gram, whereas the other groups all were on plain food.

To establish the distribution of copper among tissues and organs both counting and autoradiographic techniques were applied. For counting

larvae were dissected into fractions consisting of the middle midgut, the rest of the gut, and the remainder of the larva. In some instances the gut was further subdivided and the malpighian tubules were separated. Since considerable difficulty is entailed in recovering the malpighian tubules *in toto* they were usually included with the gut remnant instead of being counted separately.

The histochemical investigations cited above had indicated that while there is a more or less constant quantity of detectable copper in malpighian tubule cells, most of the copper which can be revealed in copper-fed larvae is located in the cells of a small section of the middle midgut. From the counting data set out in table 5, it may be seen that this predominance in histochemical copper was illusory. Doubtless, the sources of error in this

TABLE 5

CU UPTAKE BY DROSOPHILA LARVAE AND ITS DISTRIBUTION IN RELATION TO CU LEVEL

SPECIES	CU IN MEDIUM, μG./G.	μG. CU/LARVA AFTER 8 HRS. ON AND 4–8 HRS. OFF	% IN MID-MID-GUT	% IN REST OF GUT	% IN REST OF LARVA
Repleta	0.25	10×10^{-4}	16	56	27
	2.5	105×10^{-4}	13.3	45.7	41
	100	1406×10^{-4}	38.0	41	21
	200	2260×10^{-4}	43.0	37.7	19.2
Ananassae	0.25	5×10^{-4}	36.4	40.9	22.7
	2.5	40×10^{-4}	20.0	54.0	26.0
	100	857×10^{-4}	27.8	46.5	24.9
	200	604×10^{-4}	20.4	45.8	33.8
Melanogaster	0.25	4×10^{-4}	(64.8	17.6	17.6)[a]
	2.5	31×10^{-4}	14.7	39.3	45.9
	100	762×10^{-4}	18.8	59.8	18.4
	200	414×10^{-4}	24.6	63.7	11.7

[a] Very little data.

case were both concentration (that only in this region was the copper concentrated enough to give a visible color reaction) and masking (that a larger part of the non-middle mid-gut copper is in combinations not reactive in the test used). In the case of the *repleta* larvae the counting rates were enough above background and the statistical significance high enough to provide complete confidence in the reality of the increase in proportionate amount of middle mid-gut copper, as the copper supply in the medium was increased from 2.5 to 200 μg. per gram. In the cases of *ananassae* and *melanogaster* this increase is also apparent from 2.5 to 100 μg. Cu per gram. Further data are needed to establish the difference between ananassae and melanogaster at 200 μg. Cu per gram, in that even though both show toxic effects by reduction in Cu intake, in the former the middle mid-gut fraction has also been reduced whereas in the latter it has continued to increase.

Although the counting rates were quite low, we are inclined to regard as real, the uniformly greater proportion of middle mid-gut copper in larvae on 0.25 μg. as opposed to 2.5 μg. per gram.

In Figure 1 are illustrated representative whole-mount autoradiographs of dissected larval guts. In these the photographic exposure time was uniform so that differences in degree of blackening closely approximate differences in content of Cu^{64}. It is evident that small amounts of copper

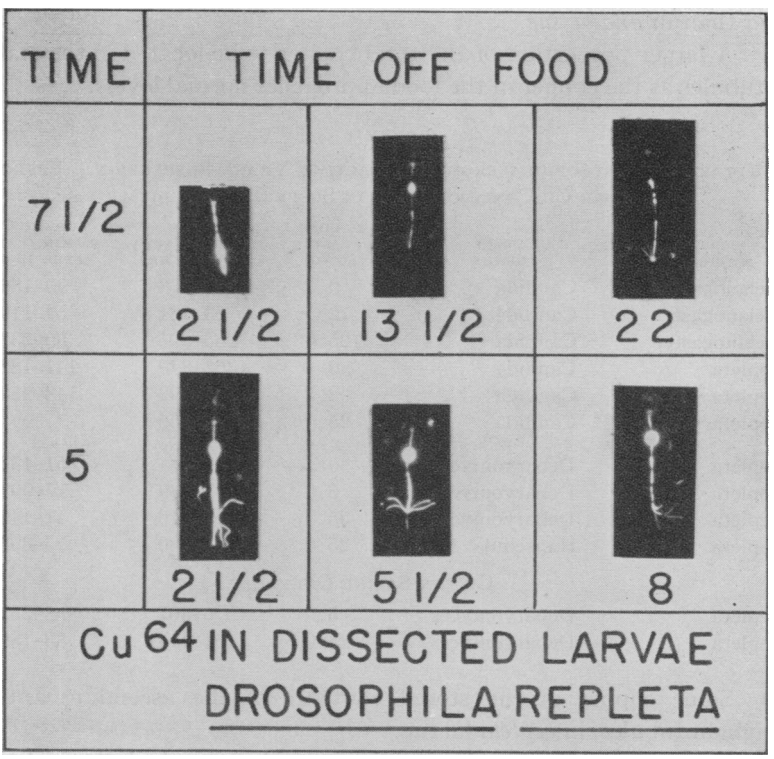

FIGURE 1

Positive prints of contact autoradiographs showing Cu^{64} in gut epithelium and Malpighian tubules. Anterior ends above, $^{8}/_{10}$ natural size.

are distributed throughout the dissections even in the distal portions of the caeca and in the malpighian tubules although the greatest concentration is clearly in the middle mid-gut. It is planned to present elsewhere an extended discussion of our combined histochemical and histological and contact autoradiographic studies. The data presented here, however, require to be supplemented with the statement that these other studies indicate:

(1) Copper excretion to be largely, if not exclusively, from the cells of the middle mid-gut. The excreted copper is not found to penetrate the peritrophic membrane but passes slowly back to the hind-gut within the space between the peritrophic membrane and gut epithelium. The behavior of copper in this space below the middle mid-gut, as had that of barium above the middle mid-gut, indicates a slow posteriad flow of fluid between peritrophic membrane and mid-gut epithelium. This middle mid-gut excretion continues over a much longer span of time in larvae of *repleta* than in *ananassae*.

(2) A larger proportion of the total copper to be located in the malpighian tubules as the copper in the food approaches normal levels.

TABLE 6

Cu^{64} UPTAKE BY DROSOPHILA FROM RADIOACTIVE YEAST SUSPENSION. EFFECT OF STABLE Cu CONCENTRATION IN SUSPENDING MEDIUM

DROSOPHILA SPECIES	YEAST GENUS	CU CONC. IN SALINE, μG./G.	RANGE, C./MIN./LARVA AFTER 10 HRS.	RANGE, C./MIN./LARVA, AFTER 19 HRS.
Melanogaster	Candida	0	117–578	90–186
Melanogaster	Candida	2.5	63–121	54–119
Melanogaster	Candida	25.0	285–395	89–237
Repleta	Candida	0	365–840	141–482
Repleta	Candida	2.5	226–692	148–953
Repleta	Candida	25.0	368–459	....
Repleta	Debaryomyces	0	20–68	51–137
Repleta	Debaryomyces	5.0	37–139	17–99
Repleta	Debaryomyces	25.0	9–116	11–149
Repleta	Hansenula	25.0	11–480	15–396
		Cu^{64} IN SALINE ONLY		
Repleta	Debaryomyces	5.0	6–307	8–252
Repleta	Debaryomyces	25.0	151–1141	83–1398

(3) Some copper in the stored contents of the ascending anterior malpighian tubules, in *repleta* larvae.

(4) The distribution of copper to tissues aside from the intestinal tract, to be extremely sparing at low copper uptake, and never very large.

In another series of experiments we investigated the larval uptake of copper contained in yeast cells. Cu^{64} was added to the liquid media on which yeasts of the genera *Candida*, *Hansenula* and *Debaryomyces* were growing and after time for uptake, the yeast cells were centrifuged out, washed with distilled water and resuspended in saline solution. This suspension was placed on filter paper and the larvae allowed to feed on the cells. The data presented in table 6 indicate not only that there was rapid and considerable absorption of Cu^{64} from the bodies of ingested yeast, but that in some way this Cu^{64} is absorbed without mixing with stable copper added to the saline in which the yeasts were suspended.

The lower lines of table 6 indicate that Cu^{64} in saline with non-radioactive yeasts is readily absorbed by the larvae. Table 2 has already presented data to show that at 25μg. Cu per gram, absorption is no longer proportional to concentration, so that it does not appear possible that the lack of dilution effect shown in table 6 may be explained by uptake of all the copper passing through the digestive tract.

Actually it appears that both chemical and morphological differentiation exists between yeast copper and medium (or perhaps soluble) copper. Histological autoradiographs of larvae at various times after feeding on Cu^{64} yeast show that the copper is not removed from the yeast cells until they have passed into the posterior mid-gut and that copper taken up from these is not concentrated in the middle mid-gut as "soluble" copper is.

By taking the proportionality of uptake to copper concentration in the food, established in table 2, correcting to the plateau for the time as indicated in table 3, and to zero excretion as indicated in table 4, we arrive at the following factors for copper content in late third instar larvae. These are expressed as μg. Cu per whole larva, or per mg. dry larva, per μg. Cu per gram of the medium on which the larvae have developed.

The factors are:

For repleta.....6×10^{-3} μg. Cu per larva or 6×10^{-3} per mg. dry wt.
For ananassae.....7×10^{-3} μg. Cu per larva or 14×10^{-3} per mg. dry wt.
For melanogaster...5×10^{-3} μg. Cu per larva or 14×10^{-3} per mg. dry wt.

The figure for *melanogaster* assumes that its uptake and excretion curves are quite like those of *ananassae*. Our analyses for the copper content of Drosophila media are quite variable but indicate a range in the neighborhood of 0.50 μg. Cu per gram fresh weight.

In the case of larvae raised on the two commonest fly food yeasts, *Candida* and *Saccharomyces*, it appears from other studies to be reported elsewhere, that most of the copper in the medium will be taken in as "soluble" copper, and will be metabolized as such. Thus we should expect 20% and perhaps much more of the total copper of normal larvae to be found in the cells of the "Cu band" of the middle mid-gut. It is gratifying to be able to report that these figures are well within the analytical range for copper when approached by neutron capture activation analysis, and that the arrangements are almost complete to perform the required analysis.

In other experiments, not reported in detail here, we have found that *Drosophila funebris*, a member of the subgenus Drosophila, as is *D. repleta*, has about the same coefficients for copper uptake. As discussed elsewhere[4] we have postulated on histochemical grounds a profound difference in copper metabolism between the two subgenera Drosophila and Sophophora (which includes *D. ananassae* and *D. melanogaster*). The radiocopper studies reported here support this hypothesis, as indicated above,

by showing differences in uptake, rate of excretion, and distribution within the organism, between members of the two subgenera. We hope that by extension of such studies as these to more closely related species of the genus and to some of the inter-specific and inter-racial crosses, it may be possible to cast light on the gene mechanisms controlling copper metabolism.

Summary.—The uptake of copper by larvae of four species of *Drosophila*, traced by Cu^{64}, is proportional to the copper concentration in the medium over the range $0.25–10 \times \mu g.$ Cu/g. Above this level uptake falls off. Rates of excretion as well as distribution in the tissues have also been determined. From these data factors relating copper content of larvae to copper concentration in the medium have been calculated. Both counting and autographic methods demonstrate that a large fraction of the tissue copper of Drosophila is in a form not demonstrable with presently available histochemical techniques. Further support is lent to the hypothesis, previously advanced,[4] of a profound difference in copper metabolism between two of the major subgenera (Drosophila and Sophophora) of this genus.

It is shown that Cu^{64} ingested as part of the yeast cell is absorbed without an opportunity of mixing with stable ionic copper simultaneously ingested. Thus there appear to be at least two pathways of copper uptake, one for ionic, the other for bound forms.

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[8] The Cu^{64} used in the studies at Yale was obtained on allocation from the U. S. Atomic Energy Commission, some samples after irradiation at Oak Ridge National Laboratory, some at Brookhaven National Laboratory.

[9] The medium used for Drosophila culture in these experiments, essentially that used

at California Institute of Technology, contains before cooking, for 100 parts water, 0.885 part Agar; 10.6 parts molasses; 8.85 parts yellow cornmeal; 1.24 parts dry Brewer's yeast; and 0.53 part propionic acid. This is cooked to remove about 29 parts water and poured into bottles.

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CONFORMAL RELATIVITY

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If one keeps invariant angle but drops invariant (four-dimensional) length from an essentially Einsteinian description of the world, one gets Conformal Relativity. This theory, skeletonized below, will be published elsewhere.

The basic idea is to let the invariance of the light cone alone (without the additional requirement that there exist an invariant length) define the class of *preferred systems* (i.e., those observers for whom the nature laws are "in their simplest form"). The field equations of the resulting theory thus exhibit *invariance in form* under the whole conformal group in local space-time, interpreted as coordinate transformation group. Accordingly, we define the *Special Theory of Conformal Relativity* to be the study of the ordinary conformal ("conformal flat") space C_4 with angle-defining form of signature $(+++-)$ 1 when the four-dimensional manifold is interpreted as space-time. It treats the kinematical relationship between equivalent observers and the behavior of measuring apparatus in the absence of all "force" fields.

The mathematical techniques needed are classical, and some of the results are already in the literature. The main result is due to J. Haantjes[1] who in 1940 discovered that the conformal group represents transformations between all observers (cartesian systems in different R_4's) in *uniform* relative acceleration. All relative velocities are bounded above by light velocity, which is of course the same for all observers. For details on the non-invariance of length and rest mass,[2] see Haantjes *loc. cit.* Other results proper to the Special Theory are, e.g. the behavior of meter sticks and clocks under conformal transformations, the composition law of relative accelerations, etc.

An *event* of this theory is defined to be a hypersphere (center x^m ($m = 1, \ldots 4$), radius R), the subclass of nullspheres[3] ($R = 0$), identified with their center points x^m, corresponding 1-1 with the events of ordinary relativity. Hexaspherical coordinates,[4] the preferred systems of C_4, refer these events