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The Effect of Inhibitors *in vivo* on Protein Synthesis and the Amino Acid Pool in the Sheep Blowfly, *Lucilia cuprina*

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1. The rates of detoxification of cycloheximide (33 μg/g fresh wt.), puromycin (167 μg/g fresh wt.) and actinomycin D (1 μg/g fresh wt.) were assessed *in vivo* on the basis of acid-insoluble [^{14}C]leucine incorporation in the sheep blowfly, *Lucilla cuprina*; these were compared with quantitative estimates which took account not only of incorporation data but also of leucine pool size and turnover. Quantitatively, cycloheximide and puromycin were still at least 50% effective in inhibiting protein synthesis after 6.5 and 24.5h of exposure respectively, whereas values based only on incorporation data suggested that cycloheximide was 83% effective and puromycin completely ineffective after the respective periods. Quantitative estimates also showed that actinomycin D effectiveness increased with increasing exposure over 24.5h, in contrast with values based only on incorporation data, which suggested that it was completely ineffective after 24h. 2. All inhibitors affected the dynamic state of the amino acid pool; there was a marked decrease in the rate of leucine-pool turnover as well as an increase in the half-life of leucine in the pool. 3. Inhibition of protein synthesis resulted in changes in leucine-pool size; the most pronounced increase occurred with cycloheximide and puromycin and the most pronounced decreases with actinomycin D. 4. Evidence is presented which suggests that proteolysis is functionally linked to protein synthesis, which determines its rate indirectly.

Inhibitors are extremely useful chemical tools for the dissection of complex cellular activities both *in vitro* and *in vivo*. However, results from such studies must be interpreted with some caution, particularly when long periods (hours, days) of exposure to the inhibitor are used as in experiments *in vivo*, for during this time the inhibitor may be detoxified considerably (see, e.g., Birt, 1971; Williams & Birt, 1972).

In studies on protein synthesis in which inhibitors are used, often little attention has been paid to the effect that inhibitors may have on changes in the size and dynamic state of the amino acid pool. Very frequently the rate of incorporation of amino acids into trichloroacetic acid-insoluble material has been equated with the rate of protein synthesis, but this is only valid if the pool size and turnover are not altered by the inhibitor.

Thus we decided to study in some detail the effect of inhibitors on protein synthesis *in vivo* and chose a dipteran, the Australian sheep blowfly (*Lucilia cuprina*) as the experimental organism, because it has several advantages. At certain times in its life cycle this insect constitutes essentially a closed system, and at particular developmental stages protein synthesis and breakdown proceed very rapidly; also there are considerable data accumulated about protein interconversions which occur during adult development (Campbell & Birt, 1972; Smith & Birt, 1972; Williams & Birt, 1972; Williams *et al.*, 1972; Campbell *et al.*, 1974), as well as evidence that there is a single pool of amino acids for protein synthesis (Williams & Birt, 1972).

The present paper reports results which allow a comparison between the effectiveness of the inhibitors cycloheximide, puromycin and actinomycin D during prolonged incubation *in vivo*. The effectiveness of these inhibitors has been evaluated on the basis of two criteria. First, the incorporation of an injected labelled amino acid into acid-insoluble material has been used as an index of protein synthesis and second, the size and turnover of the amino acid pool, as well as incorporation data, have been used to estimate protein synthesis quantitatively. Estimates of the duration of effectiveness of inhibitors are inadequate if they are based solely on the in-

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corporation of amino acids into acid-insoluble material. Evidence is also presented of a relationship between protein synthesis and proteolysis.

Experimental

Materials

Dowex 50 W (X8; 100–200 mesh), cycloheximide, puromycin dihydrochloride and actinomycin D were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and butyl-PBD [5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. [U-^{14}C]Leucine (331 mCi/mmol) and [U-^{14}C]uridine (520 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of the highest grade available.

Injection of insects

Flies (males) were reared as outlined previously (Campbell & Birt, 1972); only newly emerged (1–2h old) insects were used, to avoid complications due to any effects of the inhibitors on the emergence of the adult from the puparium (Campbell & Birt, 1972). Flies were immobilized on ice and injected at the wing base by using an apparatus described elsewhere (Campbell & Birt, 1972). Cycloheximide and puromycin were dissolved in mosquito Ringer solution (0.399 g of KCl, 7.7 g of NaCl, 0.35 g of $NaHCO_3$ and 1.1 g of glucose/litre; N. Clemens & T. D. C. Grace, unpublished work), and actinomycin D was dissolved in 95% ethanol and diluted further in water. Doses (in 0.5 μl) per insect (approx. 30 mg fresh wt.) ranged from 0.2 to 1.0 μg for cycloheximide, 1.0 to 10 μg for puromycin and 0.02 to 0.05 μg for actinomycin D; control insects received 0.5 μl of the appropriate solvent without inhibitor.

After injection, control and inhibitor-treated insects were kept at 30°C. At different times thereafter small batches (three of each) were assayed for their ability to incorporate injected [^{14}C]leucine (25 nCi/0.5 μl) or [^{14}C]uridine (50 nCi/1.0 μl) into trichloroacetic acid-insoluble material, and after 30 min at 30°C, incorporation was terminated by rapid freezing; the rate of incorporation of either label into acid-insoluble material was always linear with respect to time over this 30 min period. In some cases, at the end of the period of assessment of inhibitor effectiveness, groups of both control and inhibitor-injected insects, which had not been injected with radioisotope, were also frozen for subsequent determination of the leucine pool size.

Any losses incurred during injection and subsequent tissue processing were accounted for by measuring the total acid-soluble radioactivity remaining in newly emerged flies which had been injected with either inhibitor or inhibitor solvent, then with radioisotope and finally frozen without further incubation at 30°C. In such insects, recovery of injected radioisotope was complete (103 ± 6%). Flies kept for more than 12h at 30°C were caged and fed on sugar and water. All experiments were conducted under continuous illumination.

Processing of insects

Insects were processed singly in Eppendorf centrifuge tubes. The disintegration of the tissues and the resuspension of acid-insoluble pellets were effected by holding the centrifuge tube against a rapidly rotating rubber-coated bar. All centrifuging (15000 *g* for 1 min) was carried out at room temperature (approx. 20°C) in an Eppendorf micro-centrifuge. Each insect was dispersed in 0.5 ml of cold 10% (w/v) trichloroacetic acid and left on ice for 15 min. Acid-insoluble material was sedimented, and a sample of supernatant transferred to a scintillation vial for measurement of the radioactivity remaining in the soluble pool. The rest of the supernatant was removed, and the pellet was resuspended in 1 M-KOH, incubated at room temperature for 30 min, reprecipitated by acid, then sedimented and washed twice more with cold 10% (w/v) trichloroacetic acid. The pellet was resuspended in acid, quantitatively transferred to a glass-fibre disc (type A; Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) and washed with ethanol, ethanol–ether (1:1, v/v) and finally with ether. Discs were dried and counted for radioactivity in scintillation fluid (12 g of butyl-PBD, 800 ml of ethoxyethanol, 1200 ml of toluene) in the wide ^{14}C channel of a Beckman LS 100 counter with an efficiency of 80%.

Insects injected with [^{14}C]uridine were processed similarly, except that incubation in 1 M-KOH was replaced with a cold 10% (w/v) trichloroacetic acid wash; all centrifuging was at 4°C.

Determination of leucine pool size

An amino acid extract was prepared by homogenizing insects (four to ten) in cold 0.3 M-$HClO_4$; acid-insoluble material was sedimented by centrifuging at 27000*g* for 10 min at 4°C in the SS-34 rotor of a Sorvall Superspeed RC 2B centrifuge. The pellet was extracted twice with 0.3 M-$HClO_4$ and all acid-soluble fractions were pooled and adjusted to pH 5 to precipitate $KClO_4$, which was removed. The amino acids were adsorbed on Dowex 50 (H^+ form) and eluted with 2.5 M-NH_3, which was removed in a rotary evaporator. The sample was washed thoroughly and analysed on a Technicon amino acid auto-analyser.

Calculations

Equations to calculate the rate of turnover of amino acid in the pool ($k_{a(Leu)}$; Tables 2 and 3) and the rate of amino acid incorporation into protein ($k_{p(Leu)}$; Tables 2 and 3) were derived by Hearon (see Appendix to Dinamarca & Levenbook, 1966). They are given as $k_a=-(a/t)(\ln q_1/q_0)$ and $k_p=k_a(P)/(q_0-q_1)$, where k_a is nmol of leucine/h per insect, k_p is nmol of leucine into protein/h per insect, a is leucine pool size in nmol/insect, q_0 is injected radioactivity (55000d.p.m.), q_1 is radioactivity remaining in the soluble pool (acid-soluble fraction, 'supernatant', Table 1) after time t, P is radioactivity into total protein (acid-insoluble fraction, 'pellet', Table 1), and t is time in h. From values for $k_{a(Leu)}$ and pool size (a), it was possible to calculate the half-life of leucine in the pool (Table 4) from the following: half-life of leucine (h)$=0.693a/k_{a(Leu)}$.

Results

'Detoxification' based on incorporation of [^{14}C]leucine into acid-insoluble material

Preliminary experiments based only on incorporation data were conducted to select suitable doses of inhibitors and appropriate periods of exposure for a more detailed study of their effect; experiments using the final doses chosen are shown in Table 1.

The 'percentage detoxification' was measured (Table 1) by expressing the incorporation of radioactivity into acid-insoluble material ('pellet') of inhibitor-injected insects as a percentage of that into pellets from control insects. Thus 0% represents complete inhibition and 100% complete detoxification of inhibitor; values greater than 100% reflect a stimulation of the incorporation into pellets from inhibitor-injected insects and are referred to as showing an 'overshoot'. The radioactivity remaining in the acid-soluble fraction ('supernatant') has been expressed in the same way as for pellet radioactivity and is included for comparison with the pellet data. Incorporation of radioactivity into pellets of inhibitor-injected insects is shown in parentheses in Table 1; unlike the other values in Table 1 these are not compared with controls, and they show that 'detoxification' was due to a change in the incorporation pattern of inhibitor-injected insects (e.g. 21-fold increase after 12h with cycloheximide; Table 1) rather than to a change in the incorporation pattern of the controls. Similarly, the values in parentheses for the supernatants refer to the percentage of injected radioactivity remaining in these fractions in inhibitor-injected insects only.

In all experiments, the two protein-synthesis inhibitors cycloheximide and puromycin produced at once an inhibition of acid-insoluble incorporation of [^{14}C]leucine which was maximal or close to maximal. Thereafter a progressive decrease in inhibition was observed and this resulted finally, with cycloheximide, in a substantial overshoot in which acid-insoluble incorporation in inhibitor-injected insects was considerably larger than in control insects. Doses of each inhibitor lower than those in Table 1 produced less inhibition initially; they also resulted in a faster recovery from inhibition, e.g. with 0.2μg and 1μg of cycloheximide per insect, acid-insoluble incorporation reached 50% of the control incorporation after

Table 1. *Effect of duration of exposure to metabolic inhibitors on [^{14}C]leucine incorporation into acid-insoluble and -soluble fractions*

Groups of insects were injected with inhibitor; controls received the appropriate inhibitor solvent only. The effectiveness of the inhibitor after different exposure periods at 30°C was assessed by measuring the incorporation of [^{14}C]leucine into acid-insoluble (pellet) and acid-soluble (supernatant) material as described in the Experimental section. The values shown represent the average determinations of individual treatments to three inhibitor-injected and three control insects. The times of exposure to the inhibitor are inclusive of the radioactive assay. Doses of inhibitor per insect were 1μg of cycloheximide or 5μg of puromycin or 0.03μg of actinomycin D. Values in parentheses represent the percentage of injected radioactivity (55000d.p.m.) recovered in the respective fractions of inhibitor-injected insects only.

$$100\times\frac{\text{Radioactivity in fractions of inhibitor-injected insects}}{\text{radioactivity in fractions of controls}}\ (\%)$$

Time of exposure to inhibitor (h)	Pellet			Supernatant		
	Cycloheximide	Puromycin	Actinomycin D	Cycloheximide	Puromycin	Actinomycin D
0.5	4.2$_{(0.76)}$	—	—	176$_{(95)}$	—	—
3.5	11$_{(2.0)}$	18$_{(3.5)}$	75$_{(15)}$	186$_{(95)}$	188$_{(90)}$	135$_{(65)}$
6.5	17$_{(3.3)}$	17$_{(3.3)}$	38$_{(7.5)}$	178$_{(89)}$	170$_{(92)}$	146$_{(79)}$
12.0	100$_{(16)}$	35$_{(5.7)}$	35$_{(6.6)}$	98$_{(50)}$	162$_{(86)}$	162$_{(86)}$
24.5	160$_{(16)}$	107$_{(12.8)}$	100$_{(12.0)}$	72$_{(46)}$	99$_{(69)}$	100$_{(70)}$

4h and 9–11h respectively (9.5h, Table 1) and with 1 μg and 5 μg of puromycin per insect, the same inhibition was reached after 4.5h and 10.5–14h (14h, Table 1). The time at which acid-insoluble incorporation in inhibitor-injected insects reached 50% of the control incorporation was determined graphically from data, some of which are given in Table 1. Finally, with lower doses of cycloheximide, the overshoot of incorporation occurred more quickly, e.g. after 10.5h with 0.2 μg of cycloheximide per insect, compared with 24.5h with 1 μg (see Table 1 for 1 μg of cycloheximide). A higher dose of puromycin (10 μg per insect) than that reported in Table 1 was tested and resulted initially in an almost complete inhibition (94%), which was comparable in magnitude with that observed with 1 μg of cycloheximide per insect (Table 1). After 22.5h, incorporation into acid-insoluble material was still inhibited by 70%. However, contrary to the results of the above experiments (cycloheximide, 0.2–1 μg per insect; puromycin, 1–5 μg per insect), in which there was no mortality, with 10 μg of puromycin per insect, 27% of the injected insects died within 24h.

In contrast with puromycin and cycloheximide, actinomycin D consistently produced a small initial stimulation in incorporation into acid-insoluble material, e.g. after 0.5h there was a 17% increase over control incorporation with 0.02 μg of actinomycin D per insect and 11% with 0.04 μg per insect. Subsequently, incorporation declined to a broad minimum between 6 and 9h after injection of the inhibitor, i.e. 41% inhibition with 0.02 μg of actinomycin D per insect after 6.5h, and greater than 65% inhibition after 9h with 0.03 μg (Table 1). Thereafter incorporation increased to control values, and in a number of experiments an overshoot of incorporation in inhibitor-injected insects over that in controls was observed; however, the magnitude and time of occurrence of this overshoot varied considerably, e.g. with 0.02 μg of actinomycin D per insect the overshoot ranged from 112 to 223% and occurred between 12.5 and 30.5h after initial injection of inhibitor.

Actinomycin D inhibition of acid-insoluble [^{14}C]-uridine incorporation was initially greater than that of [^{14}C]leucine, e.g. 47% inhibition after 0.5h with 0.02 μg of actinomycin D per insect (compare above with [^{14}C]leucine) and 56% with 0.05 μg of actinomycin D; inhibition increased to a maximum of 67% after about 6.5h with 0.02 μg of actinomycin D per insect and 88% with the higher dose (0.05 μg per insect). With 0.02 μg of actinomycin D injected per insect, the inhibition of acid-insoluble [^{14}C]uridine incorporation was relieved progressively after 6.5h, whereas inhibition was still greater than 80% after 30.5h of exposure to 0.05 μg of actinomycin D per insect; however, during this 30h period, with the higher dose, 12% of the injected flies died.

Table 2. *Quantitative assessment of effect of cycloheximide, puromycin and actinomycin D on protein synthesis*

Equations to calculate the rate of turnover of amino acid in the pool (k_a) and the rate of amino acid incorporation into protein (k_p) are given in the text; other parameters (q_1 and P, see the text) used in these calculations were derived from Table 1. Pool size was determined on insects from the same group as that from which some were taken for radioactive assessment of the effectiveness of the inhibitors (see the legend to Table 1). Doses of inhibitor per insect were 1 μg of cycloheximide or 5 μg of puromycin or 0.03 μg of actinomycin D.

Time of exposure to inhibitor (h)	Pool size (nmol of leucine/insect)				k_a (nmol of leucine/h per insect)				k_p (nmol of leucine into protein/h per insect)			
	Control	Cyclo-heximide	Puro-mycin	Actino-mycin D	Control	Cyclo-heximide	Puro-mycin	Actino-mycin D	Control	Cyclo-heximide	Puro-mycin	Actino-mycin D
0.5	23	36	—	—	28	4	—	—	11.1	0.6	—	—
3.5	22	84	56	—	29	9	11	—	10.5	3.5	4.0	—
6.5	21	73	60	33	29	17	10	15	10.9	4.9	4.2	5.5
12.0	19	25	49	30	26	35	15	9	8.3	11.2	6.1	4.2
24.5	—	—	16	5	—	—	12	4	—	—	5.0	1.6

Effect of inhibitors on leucine pool size

As the rates of detoxification of inhibitors based only on incorporation of [^{14}C]leucine could be influenced by changes in leucine pool size, the effect of inhibitors of protein synthesis on the concentration of this amino acid in the pool was investigated. With each inhibitor, changes in leucine pool size relative to controls were found (Table 2). After only 30min of exposure to cycloheximide (1μg per insect), the leucine pool size had increased by 57% over that in controls (Table 2). Overall, a fourfold increase was observed with cycloheximide after 3.5h (Table 2), compared with a threefold increase with puromycin (5μg per insect), which took longer to develop (6.5h; Table 2) than with cycloheximide. At this stage, in both cases the inhibition of [^{14}C]leucine incorporation into the pellet was greater than 80% (Table 1). Subsequently, in insects injected with either inhibitor, the free leucine concentration declined to values close to or below those in the controls (Table 2). This 'cycle' was completed after 12h exposure to cycloheximide (Table 2); after 24h the leucine concentration in the pool of puromycin-injected insects was half that of corresponding controls (Table 2).

With actinomycin D (Table 2), the leucine pool concentration had increased by about 60% over that of corresponding controls when the inhibition of incorporation into the pellet was greatest (62–65% at 6.5 and 12h; Table 1). The most dramatic variation was that observed after 24h, when the leucine concentration was 15% of that of controls.

Effect of inhibitors on leucine pool turnover

From the changes in leucine concentration and in the radioactivity remaining in the supernatant fraction after 30min (Table 1), it was possible to calculate $k_{a(Leu)}$, the rate of turnover of leucine in the pool, by using the equation of Hearon given in Dinamarca & Levenbook (1966) (see the Experimental section). When incorporation into the pellet was strongly inhibited by cycloheximide (0.5–6.5h; Table 1) or by puromycin (3.5–12h; Table 1), $k_{a(Leu)}$ values were considerably lower than those of the controls (Table 2); the rapidity of this effect on the pool is clear from the changes observed even after 30min exposure to cycloheximide (Table 2). With actinomycin D, it was found that the $k_{a(Leu)}$ declined with increasing periods of exposure to the inhibitor, being lowest (Table 2) when the inhibition of incorporation was abolished after 24h of exposure (Table 1).

The dramatic decline in pool turnover is also reflected by the increases in the half-life of leucine in the pool (Table 3). With cycloheximide, puromycin and actinomycin D, the half-life of leucine in the pool was greatest after 3.5h, 3.5–6.5h and 12h of exposure to each inhibitor respectively and had increased over that in controls by 12-fold (cycloheximide), 7.5-fold (puromycin) and fourfold (actinomycin D).

Comparison of estimates of detoxification based only on incorporation of [^{14}C]leucine into acid-insoluble material with those based on both incorporation data and pool-size changes

As detoxification is the manifestation of the loss of active inhibitor, it can be determined most readily in useful terms by measuring the changes in protein synthesis after different periods of exposure to the particular inhibitor. In view of the effects of the inhibitors studied on amino acid pool size and turnover, these parameters must be taken into account together with the incorporation data when calculating protein synthesis. Accordingly, the rate of protein synthesis, k_p, expressed as the rate of incorporation of an amino acid into protein, was calculated from $k_{a(Leu)}$ and from values of [^{14}C]leucine incorporation into protein (pellet; Table 1) by using Hearon's equation (Dinamarca & Levenbook, 1966; see the Experimental section). For the purposes of this presentation, 'protein synthesis' refers to estimates which have taken account of incorporation data as well as amino acid pool size and turnover; on the

Table 3. *Effect of metabolic inhibitors on the dynamic state of the leucine pool*

The data were derived from Tables 1 and 2 by using the equation described in the text. Doses of inhibitor per insect were 1μg of cycloheximide or 5μg of puromycin or 0.03μg of actinomycin D.

Time of exposure to inhibitor (h)	Half-life of leucine in the pool (h)				
	Control	+Cycloheximide	Control	+Puromycin	+Actinomycin D
0.5	0.57	6.3	—	—	—
3.5	0.53	6.3	0.47	3.5	—
6.5	0.50	3.0	0.55	4.1	1.5
12.0	0.51	0.5	0.55	2.2	2.3
24.5	—	—	0.95	0.92	0.87

Table 4. *Comparison of the assessment of duration of effectiveness of metabolic inhibitors in vivo based on [^{14}C]leucine incorporation into acid-insoluble material with that based on estimated protein synthesis*

Inhibitions were calculated from values based on [^{14}C]leucine incorporation into acid-insoluble material (pellet; Table 1) and those based on k_p, the rate of protein synthesis (nmol of leucine incorporated into protein/h per insect; Table 2). Doses of inhibitor per insect were 1 μg of cycloheximide or 5 μg of puromycin or 0.03 μg of actinomycin D.

Time of exposure to inhibitor (h)	Inhibition (%)					
	[^{14}C]Leucine incorporation into pellet			Protein synthesis		
	Cycloheximide	Puromycin	Actinomycin D	Cycloheximide	Puromycin	Actinomycin D
0.5	96	—	—	95	—	—
3.5	89	82	25	67	64	—
6.5	83	83	62	55	61	49
12.0	0	65	65	0	36	56
24.5	—	0	0	—	48	83

Table 5. *Effect of inhibitors of protein synthesis on leucine pool size*

Measured changes in leucine pool size were estimated from the differences in the pool size values of inhibitor- and control-injected insects in Table 2. Calculated changes were estimated from the difference between the total amount of leucine incorporated into the protein of control insects (i.e. $k_{p(control)} \times t$) and that incorporated into the protein of inhibitor-injected insects (i.e. $k_{p\ (inhibited)} \times t$) over a time-period t. Each k_p value used was the average of that at the beginning and end of each of the periods (t) studied. Doses of inhibitor per insect were 1 μg of cycloheximide or 5 μg of puromycin or 0.03 μg of actinomycin D.

Period of exposure to inhibitor, t (h)	Change in pool size in presence of inhibitor (nmol of leucine/insect)					
	Cycloheximide		Puromycin		Actinomycin D	
	Measured	Calculated	Measured	Calculated	Measured	Calculated
0.5–3.5	+48	+26	—	—	—	—
3.5–6.5	−11	+20	+4	+20	—	—
6.5–12.0	−48	+8	−11	+27	−3	+29
12.0–24.5	—	—	−33	+50	−25	+84
Total	−11	+54	−40	+97	−28	+113

other hand, incorporation data alone refer to those studies in which no such account has been made of the size and dynamic state of the amino acid pool.

From the rates of protein synthesis in both control, and inhibitor-injected insects, the extent of inhibition of protein synthesis was assessed quantitatively; this could then be compared with the extent of inhibition based only on incorporation of [^{14}C]leucine into pellets from insects from the same batch (Table 4). After exposure to cycloheximide (1 μg) for 6.5h, protein synthesis was inhibited by only 55%, whereas a greater inhibition (83%) was suggested from the incorporation studies alone (Table 4). With puromycin (5 μg), protein synthesis was still about 50% inhibited after 24h of exposure to the inhibitor; this contrasted sharply with the result obtained solely from incorporation data, which suggested that after this time the inhibitor had been completely detoxified (Table 4). With actinomycin D the inhibition of protein synthesis increased with increasing lengths of exposure (up to 24h), whereas studies based only on incorporation of radioactivity into acid-insoluble material pointed to a complete detoxification after 24h (Table 4).

Effect of inhibition of protein synthesis on proteolysis

The changes in leucine concentration in the amino acid pool of inhibitor-injected insects were estimated for successive times of incubation ('measured' value, Table 5). These can be compared with the difference between the total amount of leucine incorporated into the protein of controls (i.e. $k_{p(control)} \times t$) and that incorporated into the protein of inhibitor-injected insects (i.e. $k_{p(inhibited)} \times t$) over a particular period (t) ('calculated' value, Table 5). If there is no interaction between proteolysis and protein synthesis, the amount of amino acid which accumulates in the given period of time should be equal to this difference.

The calculation was at best semi-quantitative, but the disparity between the 'measured' and 'calculated' values is so great (Table 5) that it is hard to avoid the conclusion that amino acids do not accumulate indefinitely in the absence of protein synthesis.

Discussion

From the quantitative estimates of the amount of protein synthesis taking place *in vivo* in the presence of cycloheximide, puromycin or actinomycin D, it was found that at the doses used, cycloheximide (1 μg) was detoxified relatively rapidly, whereas puromycin (5 μg) was effective for a longer time; actinomycin D (0.03 μg) also appeared to be little detoxified over the 24h period studied. Although the calculation of the true rate of protein synthesis uses simple assumptions (i.e. that the acid-soluble amino acid pool is a valid estimate of the pool for synthesis), the fact that such calculations have accounted successfully for the synthesis of 'bulk' protein (Williams & Birt, 1972), of a protein complex (actomyosin; Campbell & Birt, 1975), and of two specific proteins [cytochrome *c* (Williams *et al.*, 1972) and α-glycerophosphate dehydrogenase (Campbell *et al.*, 1974)] in *Lucilia* gives some confidence in their use. The results in the present paper illustrate three points which may be of general relevance to inhibitor studies *in vivo* in higher organisms. First, by considering only incorporation into acid-insoluble material, the data obtained may be misleading, because no provision has been made for inhibitor-induced changes in pool size and dynamic state, e.g. from incorporation data it appeared that puromycin (5 μg per insect) was detoxified after 24h, whereas quantitative estimates which took account of amino acid pool size and turnover revealed that there was essentially no change in the effectiveness of this inhibitor. This point is of general relevance to inhibitor studies of all types, e.g. those relating to DNA and RNA synthesis. There are few studies in which any attempt has been made to measure the effect of the inhibitor on the true rate of protein synthesis *in vivo* [e.g. for actinomycin D see Regier & Kafatos (1971) and Discussion of Selman & Kafatos (1974)]. Similar problems may also arise when attempts are made to quantify radioautographic studies without consideration of pool size or dynamic state.

Secondly, the inhibitors cycloheximide, puromycin and actinomycin D all have marked effects on the size of the amino acid pool and its dynamic state. In the present study, all the inhibitors initially caused an increase in the amino acid pool size of leucine, followed by a decrease to the control value or even lower. In the presence of any of the inhibitors, pool turnover, which is very rapid in insects (Williams & Birt, 1972), almost stopped. The effect of metabolic inhibitors on amino acid pool size has also been observed in a number of other systems (reptile, Coulson & Hernandez, 1971; *Neurospora crassa*, Sebald *et al.*, 1971; embryonic chicken liver, Cowtan *et al.*, 1973; colorado beetle, *Leptinotarsa decemlineata*, Bartelink & de Kort, 1973; silk moth, *Antheraea polyphemus*, Regier & Kafatos, 1971), but little attention has been paid to the dynamic state of the pool; as stated above, this applies not only to the effect of inhibitors on protein synthesis but also to those affecting DNA and RNA synthesis.

Finally, the results suggest a relationship between protein synthesis and proteolysis. In insects, such a relationship seems to be especially important because they have very high concentrations of free amino acids, which are thought to have an important role in maintaining osmotic balance (Florkin & Jeuniaux, 1964; Fyhn & Saether, 1970). In other systems, given that the cellular constituents are constantly turning over, and that these constituents have a characteristic composition, it is also likely that there must be some integrated control linking synthesis and degradation (for reviews see Schimke & Doyle, 1970; Siekevitz, 1972). With the insect system, it is possible to design experiments to examine the nature of this relationship. Two different experimentally testable hypotheses are proposed. The first suggests that proteolysis is inhibited indirectly by protein-synthesis inhibitors as a result of feedback inhibition; this has been proposed in several other systems (Mandelstam, 1958; Schimke *et al.*, 1964; Auricchio *et al.*, 1969; Hershko & Tomkins, 1971; Lee, 1971; Segal & Haider, 1971; Woodside & Mortimore, 1972). The second hypothesis suggests that proteolytic enzymes are rapidly turned over and must be resynthesized continually, hence protein-synthesis inhibitors will prevent resynthesis and therefore stop proteolysis; support for such a hypothesis has been presented previously (Sussman, 1965; Kenney, 1967; Reel & Kenney, 1968; Levitan & Webb, 1969). In *Lucilia*, as a highly active particulate proteinase that is developmentally regulated has been characterized *in vitro* (Smith & Birt, 1972), it would be possible to examine the proteinase activity of preparations from inhibitor-injected insects in order to distinguish between the two hypotheses; the first predicts that there would be no effect on proteolytic activity and the second that the activity would disappear.

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