# Generally Recognized as Safe (GRAS) Notice

# for

# L-Methionine 90% (Trade Name: L-MetPro) as a source of Methionine in Animal Diets

Prepared for :

U.S. Food and Drug Administration

Center for Veterinary Medicine

Division of Animal Feeds

Prepared by :

CheilJedang Corporation

June 28, 2017

**PART 1 GRAS notice**,

(1) CheilJedang Corporation (CJ) is submitting a GRAS notice for the substance L-Methionine 90% as a source of methionine in animal diets.

*(2) Provide the name and address of your organization;*

**CJ CheilJedang Corporation**
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*(3) Provide the name of the notified substance, using an appropriately descriptive term;*

The common or usual name of the subject substance of this notification is "L-Methionine 90%". The product will be identified and marketed as " L-MetPro." It is a source of the essential nutrient methionine, and is specifically the L-isomer of the compound.

*(4) Describe the intended conditions of use of the notified substance, including stating whether the substance will be added to food (including drinking water) for animals in which the substance will be used; identifying the foods to which it will be added, the levels of use in such foods, and the animal species for which these foods are intended (including, when appropriate, a description of a subpopulation expected to consume the notified substance); and the purposes for which the substance will be used;*

L-Methionine 90% is to be used as an ingredient in animal feed according to current good manufacturing or feeding practice as defined in 21 C.F.R § 582.1(b) ("Substances that are generally recognized as safe"). Methionine is an essential amino acid in all animal species. Methionine will be incorporated into the diet at levels commensurate with the nutritional requirement. Therefore, the required level will be decided on a case-by-case basis by animal nutritionists, based on good feeding practice for the target species.

*(5) Inform us of the statutory basis for your conclusion of GRAS status (i.e., through scientific procedures in accordance with § 570.30(a) and (b) or through experience based on common use in animal food in accordance with § 570.30(a) and (c));*

The GRAS conclusion is based on the scientific procedures as provided in 21 CFR 570.30(a) and (b).

*(6) State your view that the notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the notified substance is GRAS under the conditions of its intended use;*

The submitter has determined that the use of L-Methionine 90%, as produced by a genetically modified *Escherichia coli* K-12, for use a nutrient in animal feed is Generally Recognized as Safe based on scientific procedure and is thus exempt from the premarket approval requirement of the Federal Food, Drug and Cosmetic Act(21 U.S.C § 301 et.seq.).

*(7) State that, if we ask to see the data and information that are the basis for your conclusion of GRAS status, either during or after our evaluation of your notice, you will:*

CJ agrees to make the data and information available to FDA; and
agrees to both of the following procedures for making the data and information available to FDA:
(A) Upon FDA request, CJ will allow FDA to review and copy the data and information during customary business hours at the address you specify for where these data and information will be available to FDA; and
(B) UponFDA request, CJ will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for FDA evaluation or on paper;

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*(8) State your view as to whether any of the data and information in Parts 2 through 7 of your GRAS notice are exempt from disclosure under the under the Freedom of Information Act, 5 U.S.C. 552 (e.g., as trade secret or as commercial or financial information that is privileged or confidential);*

Part 2 section (b) includes data that is confidential. CJ has placed that information in two appendices (Appendix 1 and 2) and that information is confidential business information.

*(9) Certify that, to the best of your knowledge, the GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to you and pertinent to the evaluation of the safety and GRAS status of the use of the substance; and*

*(10) State both the name and the position or title of the person who signs the GRAS notice.*

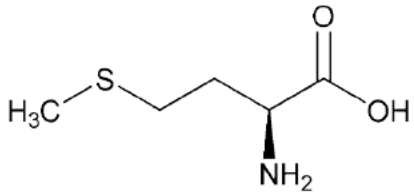
This GRAS notice is a complete, representative and balanced submission that includes unfavorable information as well as favorable information, known to CJ and pertinent to the evaluation of the safety and GRAS status of the use of L methionine 90% as produced by a genetically modified Escherichia coli K-12.

Ms. Stephanie LEE
Manager /BIO) Registration Team
CJ CheilJedang

**Part 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect.**

*(a) Scientific data and information that identifies the notified substance.*

NAME AND OTHER IDENTITIES

| | |
|---|---|
| Chemical name according to IUPAC nomenclature | L-2-amino-4-(methylthio)butanoic acid |
| Synonyms | (S)-2-amino-4-(methymercapto)butyric acid |
| CAS No. | 63-68-3 |
| EC-No. | 200-562-9 |
| Appearance | Yellowish or brown powder |
| Molecular mass | 149.21 g/mol |
| Molecular formula | $C_5H_{11}NO_2S$ |
| Structural formula |  |



L-Methionine belongs to the aspartate amino acid family. The conclusion covers L-MetPro produced by fermentation and enzyme conversion with Escherichia coli K12, with a purity of minimum of 90.0 % of L-Methionine. Due to its dedicated chemical properties, L-Methionine can only be found as free amino acid, which must not be transformed into a salt to be stable during production, storage and application.

COMPOSTION

The majority of the product is L-Methionine(≥90%). The product also consists of other amino acids (b) (4) which are consist of Glutamic acid, Alanine, Isoleucine, Leucine, Tyrosine, Phenylalanine, Threonine, homoserine.

Table1. Chemical composition including impurities.

| | Substance | Unit | Standard | Average |
|---|---|---|---|---|
| Purity | L-Methionine | % | (b) (4) | |
| Moisture | Moisture | % | | |
| Ion | $Na^+$ | % | | |
| | $NH_4^+$ | | | |
| | $K^+$ | | | |
| | $Ca^{2+}$ | | | |

| | | | |
|---|---|---|---|
| | $Mg^{2+}$ | | (b) (4) |
| | $Cl^-$ | | |
| | $PO_4^{2-}$ | | |
| | $SO_4^{2-}$ | | |
| Total ion | | | |
| Amino Acid | Glutamic acid | % | |
| | Alanine | | |
| | Isoleucine | | |
| | Leucine | | |
| | Tyrosine | | |
| | Phenylalanine | | |
| | Threonine | | |
| | homoserine | | |
| Total amino acid | | | |
| Organic acid | acetic acid | % | |
| | lactic acid | | |
| Total Organic acid | | | |
| Total of quantified components | | % | |

FERMENTATION ORGANISM

The fermentation organism is a modified Escherichia coli K-12. The modification is described in Part 2(b) and the safety of the organism is discussed Part 6.

*(b) A description of the method of manufacture of the notified substance in sufficient detail to evaluate the safety of the notified substance as manufactured;*

L-Methionine 90% is manufactured by a two-step fermentation process. Using a modified Escherichia coli K-12. (b) (4)

(b) (4) L-MetPro manufacturing process includes crystallization by concentration of mother liquid (ML) from L-methionine, product separation and drying. The final product is separated from the biomass.

The prefermentation process (genetic modification information) is provided in Appendix 1 (confidential).

The full fermentation process and other manufacturing information is provided in Appendix 2 (confidential).

Product Stability

We have tested the conducted stability tests on the L-MetPro over 12 months (Table 3). Storage condition was 25℃ ±2℃ and 60% RH. ± 5% RH. None of the tested samples showed a significant decrease in the level of the active substance L-methionine at the tested time points. The specified minimum of 90% L-methionine was maintained in all samples over the tested periods.

Table2. Shelf life of L-MetPro in % (target value is a minimum 90% L-Methionine) during storage.

| Storage time (months) | Initial | 3 | 6 | 9 | 12 | 24 |
|---|---|---|---|---|---|---|
| Batch 1 | 91.25 | 91.52 | 91.38 | 90.99 | 91.20 | - |
| Batch 2 | 91.68 | 92.01 | 91.78 | 91.88 | 91.85 | - |
| Batch 3 | 92.01 | 92.32 | 92.41 | 91.62 | 92.48 | - |
| Storage conditions: 25℃ ±2℃ and 60% RH.±5%RH | | | | | | |

*(c) Specifications for material that is of appropriate grade for use in animal food;*

The specifications are based on the assay of 5 batches. The analytical data is available upon request of the reviewers.

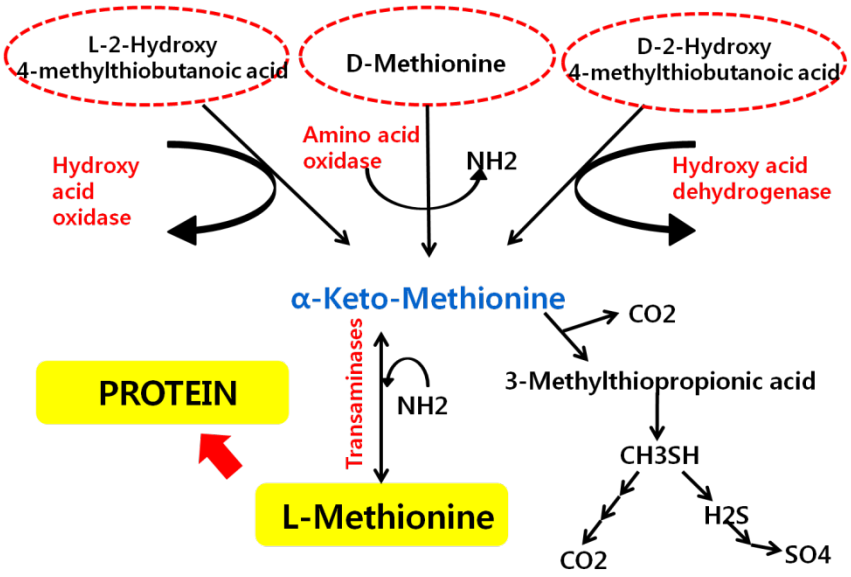
Table 3 : Specification

| Analyte | Specification |
|---|---|
| L-Methionine | Min. 90.0 % |
| Water (Loss on Drying) | Max. 1.5 % |
| Ash | Max. 1.0 % |

*(d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.*

The L-Methionine 90% is to be used is a nutrient in animal feeds in accordance with good manufacturing or feeding practice as defined in 21 CFR 582.1(b) Substances that are generally recognized as safe. Methionine exists as a stereoisomer, either as D-methionine or L-methionine. L-methionine is the physiologically relevant stereoisomer. L-methionine is an essential amino acid in all animal species (EFSA,

2012). The level of supplementation varies between species and is dependent on the nutritional content of the diet (specifically the amino acids content). Therefore, the use of supplementation will be determined on a case-by-case basis by animal nutritionists, based on good feeding practice.




Metabolism of the stereoisomers of methionine and methionine hydroxyl analogue*
* Source: British Journal of Nutrition (1989), 62, 43-75

Based on the analyses of the Methionine 90% we conclude that L-methionine produced by E. coli K-12 is the same amino acid, it is substantially equivalent the GRAS Methionine (21 CFR 582.5475) for the purpose of feed supplementation in the target animals. No component L-methionine 90% product differs significantly from the constituents of the ordinary diet of the target animal.

As L-methionine is understood to be an essential nutrient, as was found in the agency decision on AGRN 16, this product has utility as an essential nutrient.

**Part 3 of a GRAS notice: Target animal and human exposures.**

In part 3 of your GRAS notice, you *must provide data and information about exposure to the target animal and to humans consuming human food derived from food-producing animals, regardless of whether your conclusion of GRAS status is through scientific procedures or through experience based on common use in food, as follows:*
*(a) For exposure to the target animal, you must provide:*
*(1) The amount of the notified substance that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet, including the intended use and all other sources in the total diet; and*
*(2) When applicable, the amount of any other substance that is expected to be formed in or on food because of the use of the notified substance (e.g., hydrolytic products or reaction products);*
*(3) When applicable, the amount of any other substance that is present with the notified substance either naturally or due to its manufacture (e.g., contaminants or by-products);*
*(4) The data and information you rely on to establish the amount of the notified substance and the amounts of any other substance in accordance with paragraphs (a)(1) through (a)(3) of this section that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet; and*
*(b) When the intended use is in food for food-producing animals, you must provide:*
*(1) The potential quantities of any residues that humans may be exposed to in edible animal tissues, including:*
*(i) Residues of the notified substance;*
*(ii) Residues of any other substance that is expected to be formed in or on the animal food because of the use of the notified substance; and*
*(iii) Residues from any other substance that is present with the notified substance whether naturally, due to its manufacture (e.g., contaminants or by-products), or produced as a metabolite in edible animal tissues when the notified substance is consumed by a foodproducing animal; and*
*(2) The data and information you rely on to establish, in accordance with paragraph (b)(1) of this section, the potential quantities of any residues that humans may be exposed to in edible animal tissues.*

TARGET ANIMAL EXPOSURE

L-methionine is an essential amino acid in all animal species (NRC, 2012). The level of supplementation varies between species and is dependent on the nutritional content of the diet (specifically the amino acids content). Therefore the use of supplementation will be determined on a case-by-case basis by animal nutritionists, based on good feeding practice.

Based on the overall level of supplementation in the most fortified diets, (for example broilers, egg-layer, and high-producing dairy cattle), the maximum level of use methionine would in normal feeding practices be approximately 0.3% of the feed.

The impurities of this ingredient are all either essential nutrients or typical components of feed. The exposure is consistent with other feedstuffs.

HUMAN FOOD EXPOSURE

As L-methionine 90% is a source of methionine (90%) the methionine is used in the production of proteins as is all source of methionine. A such the exposure in human from all animal consuming L-methionine 90% would be that methionine incorporated in proteins of milk, meat or eggs (consumable animal products).

European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed the safety and efficacy of various methionine compounds when used in animal diets (EFSA, 2012). The EFSA Panel noted that methionine and methionine-based additives in the feed of animals result in the incorporation of all absorbed methionine in tissue protein, and methionine provided that exceeds the methionine requirement of the animal is excreted. Consequently, no free methionine occurs or accumulates in target animal tissues, and the only form L of methionine that humans will be exposed to from its use in animal feed is in the form of protein that will be digested, absorbed; and metabolized consistent with human nutrient needs. L-methionine is an essential amino for humans. Free methionine is not a residue issue. Therefore, L-methionine 90% from modified Escherichia coli K-12 presents no exposure risk to humans consuming tissues or products from the target animal.

The impurities found in the product (amino acids, minerals and organic acids) are consistent with normal components of feed, as such would not be a source of residues beyond that found in animal food products from traditionally fed animals. .

**Part 4 of a GRAS notice: Self-limiting levels of use.**
*In circumstances where the amount of the notified substance that can be added to animal food is limited because animal food containing levels of the notified substance above a particular level would become unpalatable or technologically impractical, in Part 4 of your GRAS notice you must include data and information on such self-limiting levels of use.*

There is no self-limiting use information specific to this substance.

**Part 5 of a GRAS notice: Experience based on common use in food before 1958.**

*If the statutory basis for your conclusion of GRAS status is through experience based on common use in animal food, in Part 5 of your GRAS notice you must include evidence of a substantial history of consumption of the notified substance for food use by a significant number of animals of the species to which the substance is intended to be fed prior to January 1, 1958, and evidence of a substantial history of consumption by humans consuming human foods derived from food-producing animals prior to January 1, 1958.*

The GRAS determination is not based on common use in animal feed prior to 1958.

**Part 6 of a GRAS notice: Narrative.**

*In Part 6 of your GRAS notice, you must include a narrative that provides the basis for your conclusion of GRAS status, in which:*

*(a)(1) You must explain why the data and information in your notice provide a basis for your view that the notified substance is safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food producing animals. In your explanation, you must address the safety of the notified substance, considering all animal food (including drinking water) as part of the animal's total diet, taking into account any chemically or pharmacologically related substances in such diet. In your explanation, you must also address the safety of the notified substance in regard to human exposure, considering all dietary sources and taking into account any chemically or pharmacologically related substances;*

*(2) In your explanation, you must identify what specific data and information that you discuss in accordance with paragraph (a)(1) of this section are generally available, and what specific data and information that you discuss in accordance with paragraph (a)(1) of this section are not generally available, by providing citations to the list of data and information that you include in Part 7 of your GRAS notice in accordance with § 570.255;*

*(b) You must explain how the generally available data and information that you rely on to establish safety in accordance with paragraph (a) of this section provide a basis for your conclusion that the notified substance is generally recognized, among qualified experts, to be safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from foodproducing animals;*

*c) You must either:*

*(1) Identify, discuss, and place in context, data and information that are, or may appear to be, inconsistent with your conclusion of GRAS status, regardless of whether those data and information are generally available; or*

*(2) State that you have reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with your conclusion of GRAS status;*

*(d) If you view any of the data and information in your notice as exempt from disclosure under the Freedom of Information Act, you must identify the specific data and information; and*

*(e) For non-public, safety-related data and information considered in reaching a conclusion of GRAS status, you must explain how there could be a basis for a conclusion of GRAS status if qualified experts do not have access* to such data and information.

**Safety of E. coli –Production Organism**

Escherichia coli are a Gram-negative bacteria belonging to the family of Enterobacteriaceae. Strains of Escherichia coli are part of the normal micro flora of intestines of all vertebrates (Smith, 2006). All strains of Escherichia coli used for the biotechnological production of L-Methionine are classified as Escherichia coli K12. As such they are scientifically recognized as safe bacterial strains and harmless to impacts on human and environment (Ref-1, 2). Also, Escherichia coli K12 stain is regarded as safe and QPS. Additionally they impose a long history of apparent safe use in industrial production of e.g. L-Valine and L-isoleucine (Ref-3, 4). Strains of Escherichia, actually used in the fermentative production of L-Methionine, have been improved by classical strain development implementing traditional mutagenesis, genetic modification and breeding technologies.

Members of the family of Enterobacteriaceae are Gram-negative, oxidase-negative, rod-shaped bacteria, 0.3 – 1.0 x 1.0 – 6.0 µm. Typically, they are motile by peritrichous flagella. They are facultative anaerobes, being chemo-organotrophs that exhibit both respiratory and anaerobic fermentative metabolism. Most grow on glucose as a sole carbon source, although some require vitamins and/or amino acids for growth. FDA, in their review of the determination that chymosin, the first recombinant approved enzyme for use in food was expressed in E. coli K-12 was safe (Flamm, 1991) concluded that E. coli K-12 was a nonpathogenic and nontoxigenic organisms safe for producing non-endogenous proteins.

In addition, the GRAS ingredient does not include any E. coli cells as the L-methionine is purified from the fermentation.

There are published reports of the safety of E. coli K-12, which also support the utility. For example in cattle fed E. coli K-12 with pathogenic plasmids demonstrated not adverse effects (Curtiss 1978). Even when E. coli K-12 was provided intravenously to chickens, there were no pathogenic and toxigenic effects (Smith, 1978).

**Safety of consideration due to the nature of modification to E.coli**

The strains of Escherichia coli K12 (b) (4), actually used in the fermentative production of L-Methionine, have been improved by classical strain development implementing traditional mutagenesis, genetic modification and breeding technologies.

* Escherichia coli K12 (b) (4)

(b) (4)

** Escherichia coli K12 (b) (4)

(b) (4)

Neither the production strain nor its recombinant DNA was detected in the L-methionine product. The final products do not raise any safety concern with regard to the genetic modifications. (Appendix1).

Even thus, we have used the Pariza and Cook (2010) decision tree, in an abundance of caution.

PARIZA AND COOK (2010) Decision Tree:

1. Is the production strain: genetically modified? - YES (go to 2)
2. Is the production strain modified using rDNA techniques? - YES (go to 3)
3. Below:
   a. Do the expressed enzymes (*in this case substance*) which are encoded by the introduced DNA have a history of safe use in food or feed? - YES (go to 3e)
   e. Is all other introduced DNA well characterized and free of attributes that would rended it unsafe for constructing microorganisms to be used to produce feed-grade products ? - YES (see appendix 1) (go to 4)
4. Is the introduced DNA randomly integrated in t the chromosome?
   - NO (see appendix 1) (go to 6)
6. Is the production strain sufficiently well characterized so that one may reasonably conclude that the unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed? - YES (see appendix 1)

**Accepted**

The modified E. coli K-12 lineage contains encoding sequences that are well characterized and endogenous, they are antibiotic free and the final product is free to toxins and unsafe metabolites.

## SAFETY CONSIDERATIONS OF L-METHIONINE 90%

As L-MetPro is a source of methionine (90%) the methionine is used in the production of proteins as is all source of methionine.

European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed the safety and efficacy of various methionine compounds when used in animal diets (EFSA, 2012)., The EFSA

Panel noted that methionine and methionine-based additives in the feed of animals result in the incorporation of all absorbed methionine in tissue protein, and methionine provided that exceeds the methionine requirement of the animal is excreted. Consequently, no free methionine occurs or accumulates in target animal tissues and the only form L of methionine that humans will be exposed to from its use in animal feed is in the form of protein that will be digested, absorbed; and metabolized consistent with human nutrient needs. L-methionine is an essential amino for humans. Free methionine is not a residue issue. Therefore, L-methionine 90% presents no exposure risk to humans consuming tissues or products from the target animal.

A seen in Table 1, there is no substances in the product that are not a not a typical component of feed and are nutrients.

To corroborate the safety assessment, CJ conducted an acute toxicity study in rats (appendix 3). In this acute toxicity study, following a sighting test at a dose level of 2000 mg/kg, an additional four fasted female Wistar strain rats animals were given a single oral dose of L-methionine 90%, as a suspension in arachis oil BP, at a dose level of 2000 mg/kg body weight.

Mortalities, clinical signs, body weight changes and necropsy were monitored for 14days following the administration in each test step.

The results were summarized as follows:

***Mortality.*** There were no deaths.
***Clinical Observations***. There were no signs of systemic toxicity.
***Body Weight.*** All animals showed expected gains in body weight.
***Necropsy.*** No abnormalities were noted at necropsy.

The acute oral median lethal dose (LD50) of L-methionine 90%, in the female Wistar strain rat was estimated to be greater than 2000 mg/kg body weight (Globally Harmonized Classification System Unclassified).

**Safety Assessment of known impurities and/or potential contaminants**

Based on the purity of the product, there are no known impurities or contaminants introduced in the manufacture of the product that could raise safety concerns. The product is 90%, the specifications permit (b) (4) (b) (4) the use levels of methionine in the diet are so small that you cannot consider these impurities a nutritional source of these minerals, and free amino acids at ppm levels (see section 6.B. of the submission).

Table4. Feed levels of L-methionine 90% -Impurities

| Substance | Average level in L-methinone 90%, % | Feed Level when Met Pro incorporated at 0.3%, ppm |
|---|---|---|
| $NH_4^+$ | | |
| $K^+$ | | |
| $SO_4^{2-}$ | | |
| Glutamic acid | | |
| Alanine | | |
| Isoleucine | | |
| Leucine | | |
| Tyrosine | | |
| Phenylalanine | | |
| Threonine | | |
| Homoserine | | |
| Acetic acid | | |

**SAFETY ASSESSMENT FOR HUMAN CONSUMPTION**

The L-methionine 90% is intended for use as a nutrient for animal consumption.
Ordinarily, a GRAS notice will address the potential human dietary consumption of a component of animal feed due to consumption of animal products and tissues in which the component may be present. In this case, however, there is no need to determine the estimated daily intake (EDI) of the L--methionine 90% for human consumption. The L methionine 90% and any of the described impurities (see above) will be metabolized when the animal consumes and digests its food(like all feed). The L-methionine derived from the modified E. coli K-12 will be indistinguishable from other sources, as will be the potential impurities.

In this regard, the European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed (EFSA, 2012) the safety and efficacy of methionine, methionine sodium salt, the hydroxy analogue of methionine, and the calcium salt of methionine hydroxy analogue for use in the diets of all animal species. In the report the EFSA Panel noted that methionine and methionine-based additives in the feed of animals result in the incorporation of all absorbed methionine in tissue protein. Doses exceeding the methionine requirement of the animal will be excreted. Consequently, no free methionine occurs or accumulates in target animal tissues and the only form of methionine that humans will be exposed to from its use in animal feed is in the form of protein that will be digested, absorbed; and metabolized consistent with human nutrient needs. The absence of residual methionine in the tissues of animals consuming any form of methionine in its diet will, therefore, not result in a subsequent human exposure or safety issue. As indicated by the analytical

values displayed in Table 1 and Table 3, residual components of L-methionine 90% are at levels so low as to present. No risk of humans in humans consuming the tissues of food animals fed the nutrient. All residual constituents are common metabolites or minerals and will be either excreted or metabolized. Therefore, thy present no exposure risk to humans consuming tissues or products from the target animal. A review of the publicly available literature does not reveal information demonstrating that any of these residual constituents appears to present a risk of accumulation or harm to humans at the levels that would be consumed from animal tissue. It should also be noted that L-methionine is an essential' amino for human nutrition is approved for direct addition to human food (21 CFR 172.320).

A similarly produced L-methionine product (L-methionine 85%) has been assessed by a number of acute toxicity studies, in vivo genotoxicity studies, a sub chronic oral toxicity study and a developmental toxicity study. These studies were reported to the agency as a part of Animal GRAS Notification 16, and are referenced, herein.

## SAFETY CONCLUSION

Based on the documentation provided in this GRAS Notification and as discussed above, CJ, have concluded that L-methionine 90% produced by genetically modified Escherichia coli K-12 is generally recognized as safe via scientific procedures as a nutrient for animal consumption.

**Part 7 of a GRAS notice: List of supporting data and information in your GRAS notice.**

*(a) In part 7 of your GRAS notice, you must include a list of all of the data and information that you discuss in Part 6 of your GRAS notice to provide a basis for your view that the notified substance is safe under the conditions of its intended use as described in accordance with § 570.250(a)(1).*

*(b) You must specify which data and information that you list in accordance with paragraph (a) of this section are generally available, and which data and information are not generally available*

PUBLICALLY AVAILABLE REFERENES:

Cherepanov, P.P. and W. Wackernagel, 1995. Gene disruption in *Escherichia coli*: $Tc^R$ and $Km^R$ cassettes with the option of F1p-catalyzed excision of the antibiotic-resistance determinant, Gen, 158; 9-14.

Curtis, R., 1978. Biological containment and cloning vector transmissibility. Journal of Infectious Diseases; 137 : 668-675.

Datsenko, K.A. and B.L. Wanner, 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. PNAS, June; Vol. 97, No. 12.

Doroshenko, V.G. and V.A. Livshits, 2004. Structure and mode of transposition of Tn2555 carrying sucrose utilization genes. FEMS Microbiology Letters 233(2004); 353-359.

EFSA, 2008. Efficacy and safety of L-valine from a modified E. coli K12 for all animal species. EFSA Journal; 695, I-21.

EFSA, 2010. Scientific opinion on the safety and efficacy of L-isoleucine for all animal species. EFSA Journal; 8(1):1425.

EFSA, 2012. Scientific opinion on DL-methionine, DL-methionine sodium salt, the hydroxyl analogue of methionine and the calcium salt of methionine hydroxyl analogue in all animal species; on the isopropyl ester of methionine hydroxy analogue and DL-methionine technically pure protected with copolymer vinylpyridine/styrene in dairy cows; and on DL-methionine technically pure protected with ethylcellulose in ruminant. EFSA Journal 10(3); 2623-2664.

EPA, 1994. Final decision document *Escherichia coli* K-12. TSCA Section 5(H)(4) Exemption.

Flamm, E., 1991. How FDA approved chymosin: A case history. Bio/Technology; Vol 9.

Nature Technology, 2013. pKD46-RecA and pKD46-RecApa Recombineering Vectors Instruction Manual. Nature Technology Corporation.

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## *APPENDICES*

Appendix 1 :Prefermentation Information (CONFIDENTIAL)
Appendix 2: Fermentation Process (CONFIDENTIAL)
Appendix 3: Acute Toxicity Report

## APPENDIX 1—PREFERMENTATION INFORMATION (CONFIDENTIAL)

### A. Characterization of the production microorganism

#### A-1) Scientific name and Taxonomy of the parent strain

The parent strain of methionine production strain is an Escherichia coli K12. Escherichia coli are a Gram-negative bacterium belongs to the family of Enterobacteriaceae. Strains of Escherichia coli are part of the normal micro flora of intestines of all vertebrates. All strains of Escherichia coli used for the biotechnological production of commercial amino acid are classified as Escherichia coli K12. As such they are scientifically recognized as safe bacterial strains and harmless to impacts on human and environment (ref1, ref2). Additionally they impose a long history of apparent safe use in industrial production (ref3, ref4). The strains of Escherichia coli K12 (b) (4) actually used in the fermentative production of L-Methionine, have been improved by classical strain development implementing traditional mutagenesis, genetic modification and breeding technologies.

- Scientific name: Escherichia coli k12
(To the best knowledge of the applicant, E.coli K12 has no plasmid, sex factors and prophages as Lamda and P1.)

- Taxonomy
The taxonomical position of the production strain E.coli K12 is as follow
Kingdom: Bacteria
Phylum: Scotobacteria
Order: Eubacteriales
Family: Enterobacteriaceae
Genus: Escherichia
Species: Escherichia coli
Strain: E.coli K12

Confirmation of the taxonomic position was made on the production strain using 16s rDNA sequencing (Fig 1). To the best knowledge of the applicant, the methionine production strain has no plasmid sex factors and prophages as lamda and P1. The methionine production strain does not have any antibiotic resistant genes; hence this strain does not show any antibiotic resistance.

**A-2) Nature habitai of the Ecoli K12 and its ecological role**

*E. coli* K12 does not produce spores. *Salmonella, Campylobacter, Escherichia, Shigella*, and *Vibrio* species, and species from other genera can exist in a state where they are viable but cannot be cultured by normal microbiological methods. Studies show that *E. coli* K-12 strain in soil and water show that the *E. coli* cells were disappearing from the non-sterile microcosms studied. K-12 is recognized the non-pathogenic microorganisms and can't colonize in human intestinal tract. Because E. coli iK-12 cell walls mucous membrane lack of adhesion recognition and other functions related ingredients. K-12 safety is certified by Europe and the USA and other countries safety evaluation institutions.

**A-3) Phenotypic characterisrics**

The production does not have any foreign genes or antibiotic resistant genes; hence this strain does not show any antibiotic resistance. E.coli K12 which is the parent is a facultative anaerobic micro-organism with an optimum growth temperature between 33 ~ 37 °C and an optimum growth pH between 6.5 and 7.2. This strain can grow on glucose or glycerol, but cannot utilize sucrose as a sole carbon source.

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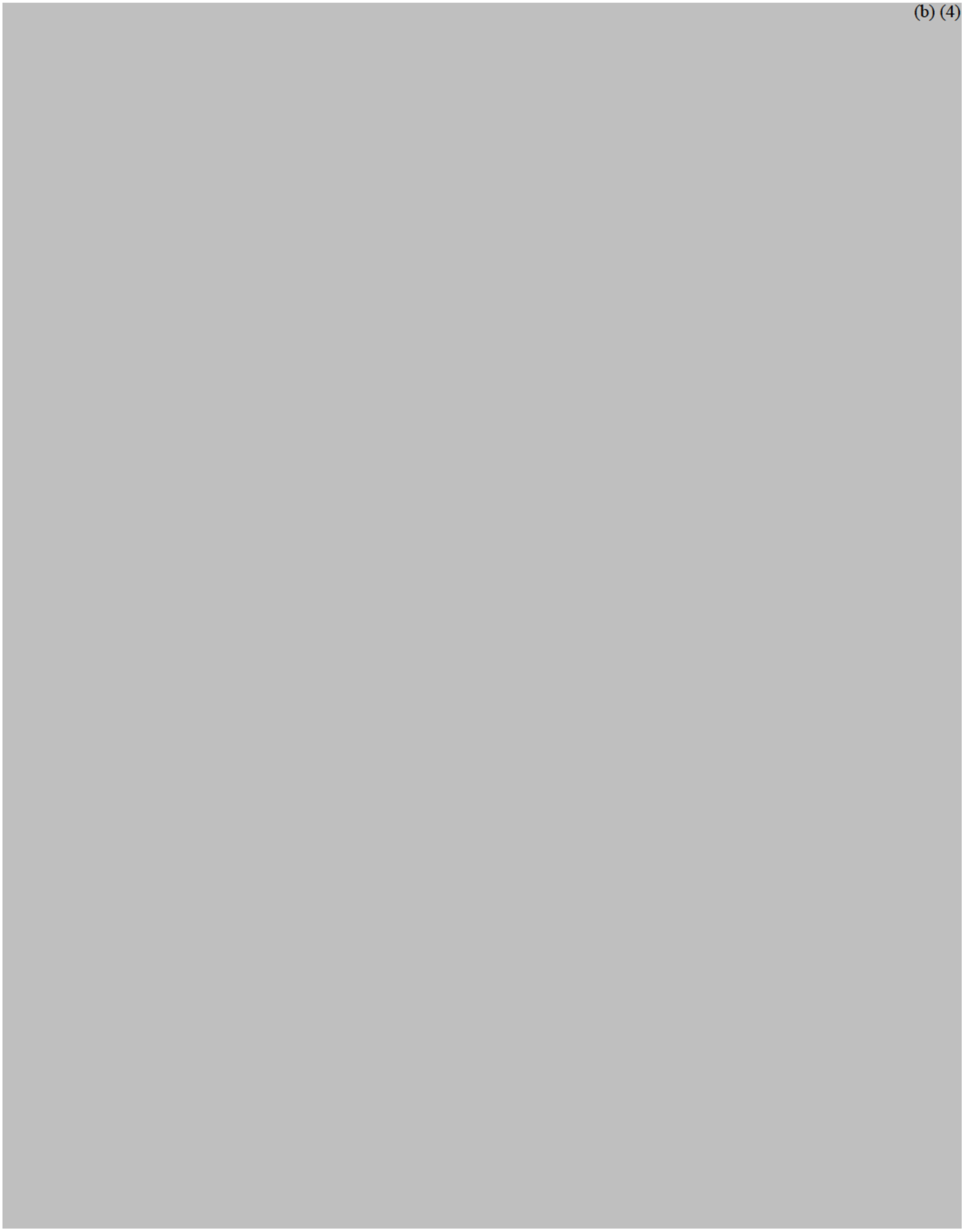

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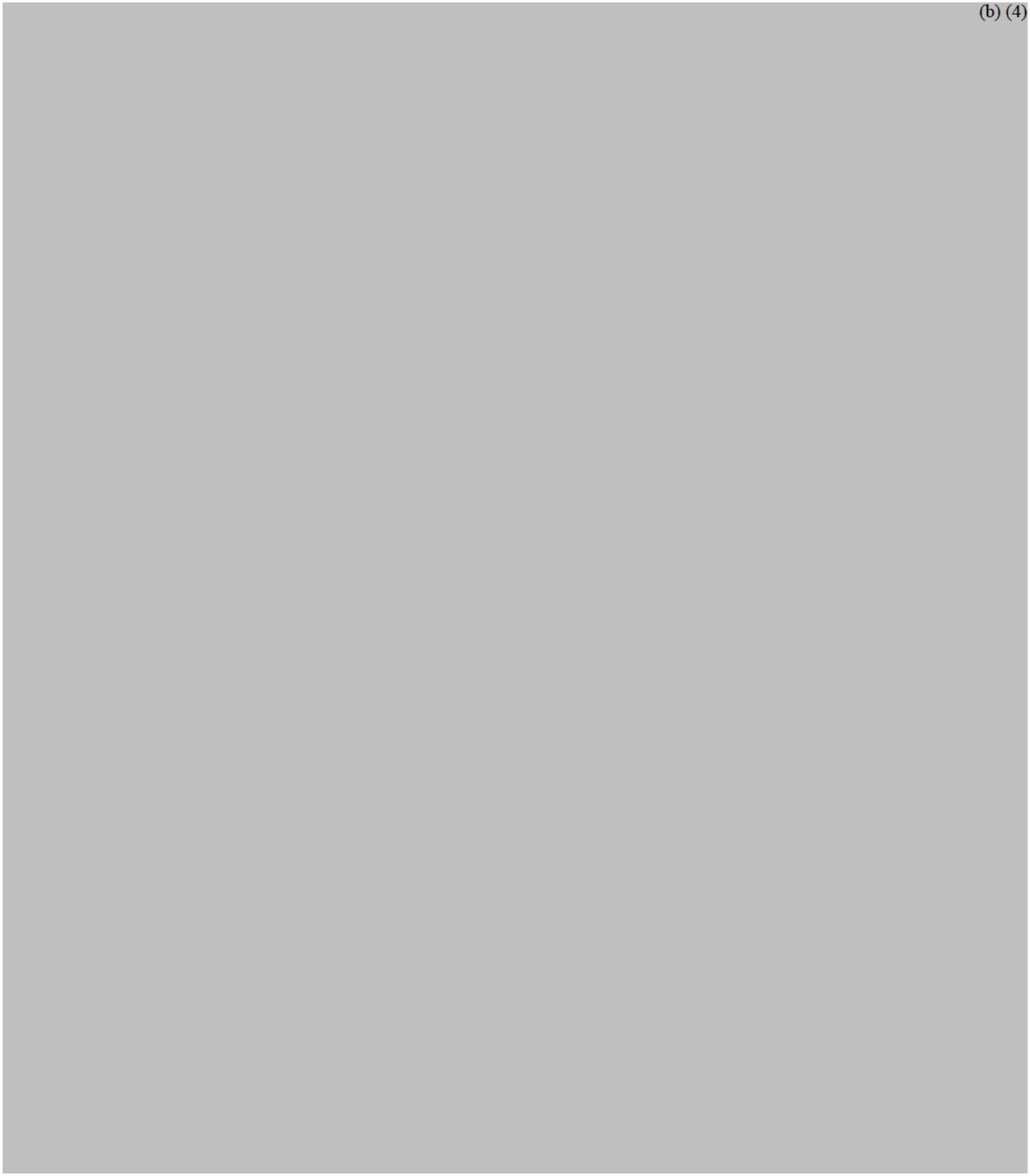

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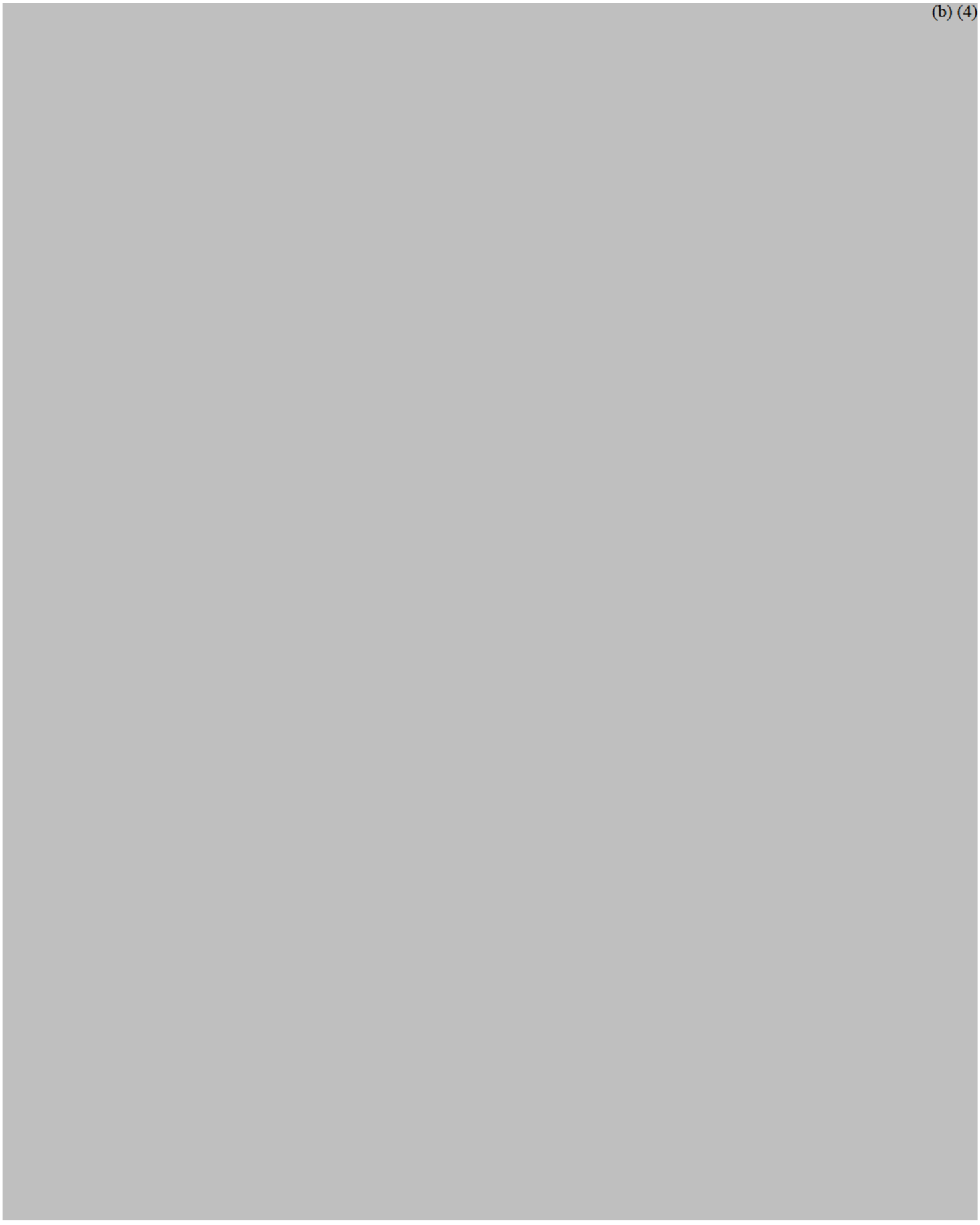

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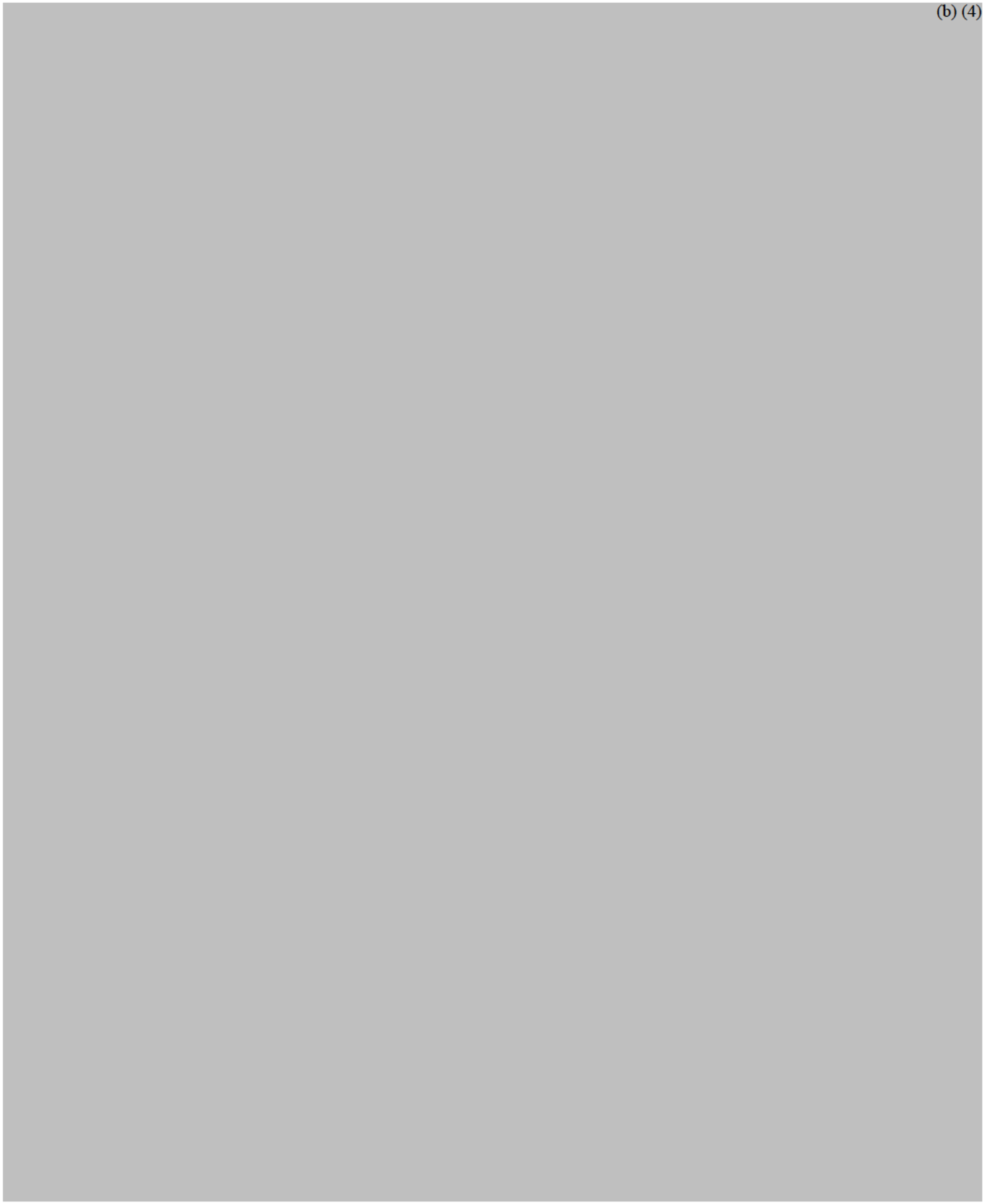

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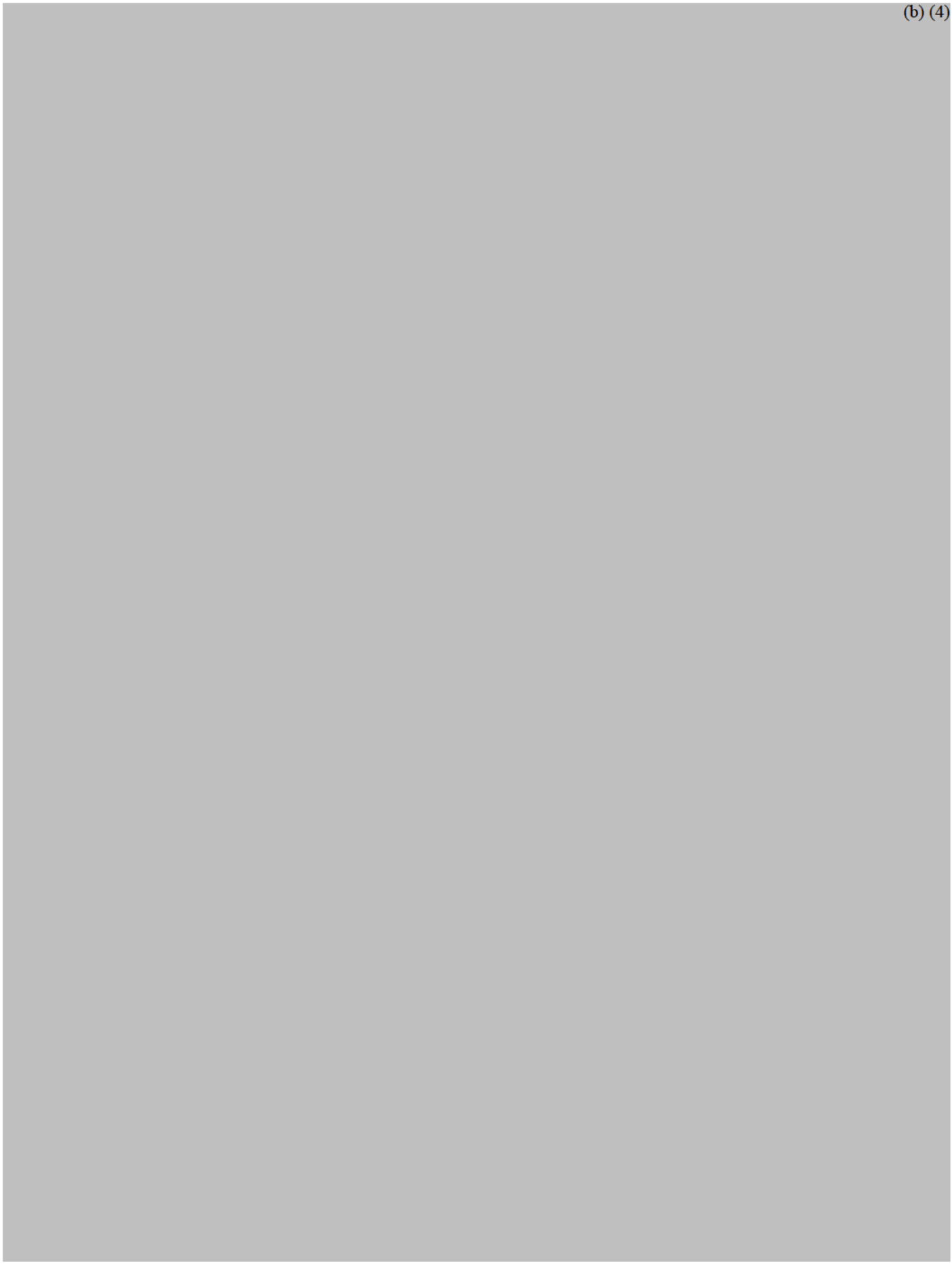

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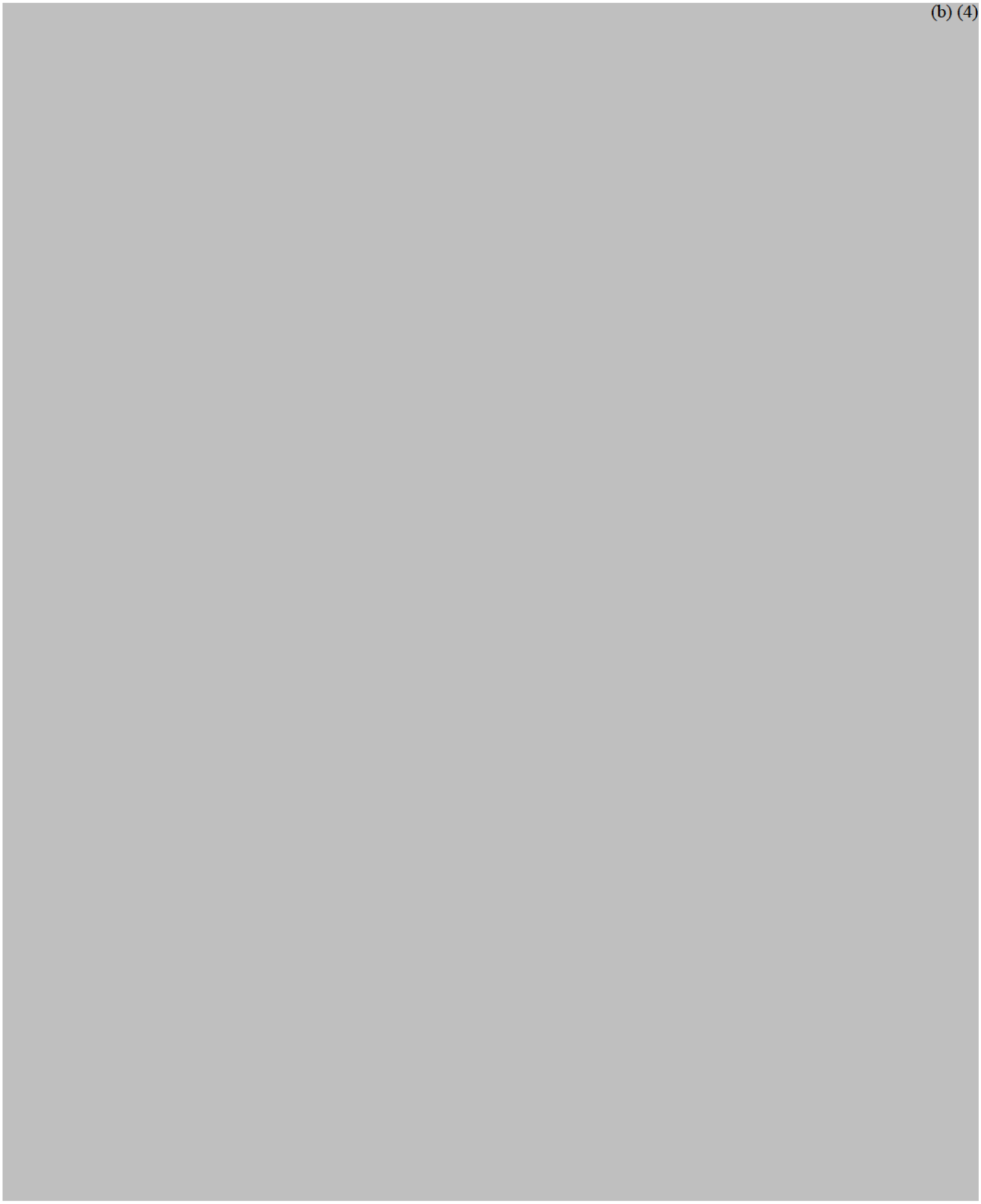

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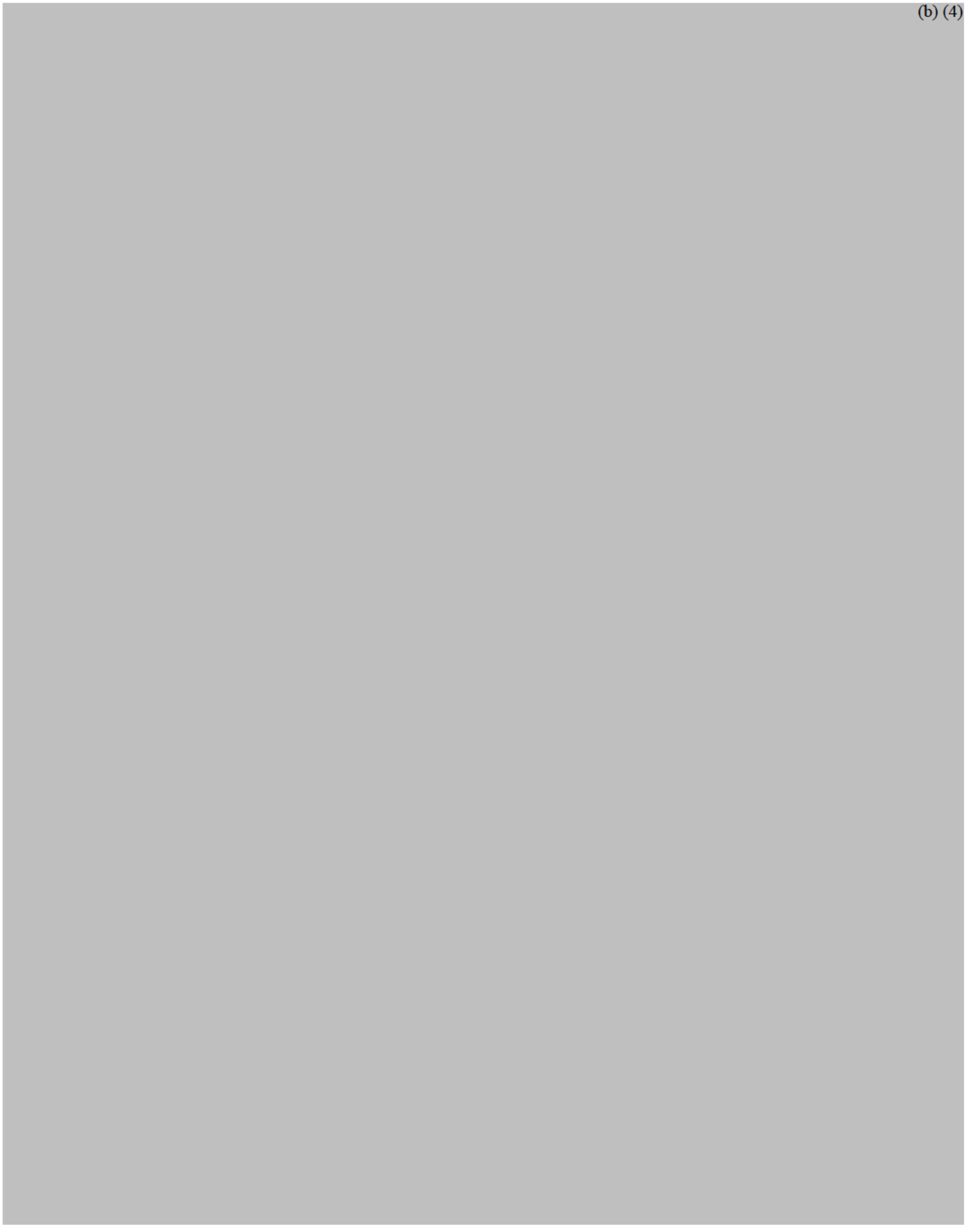

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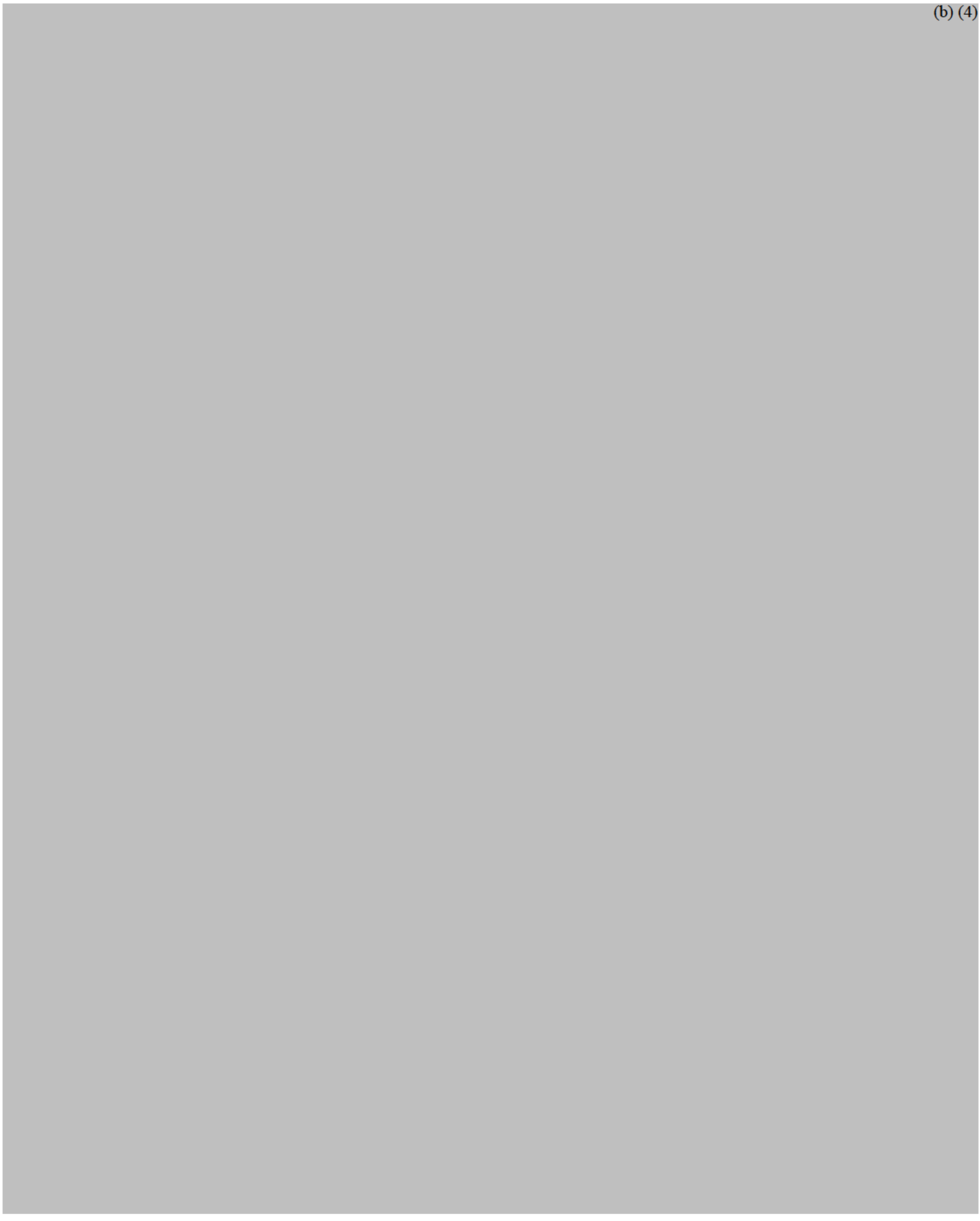

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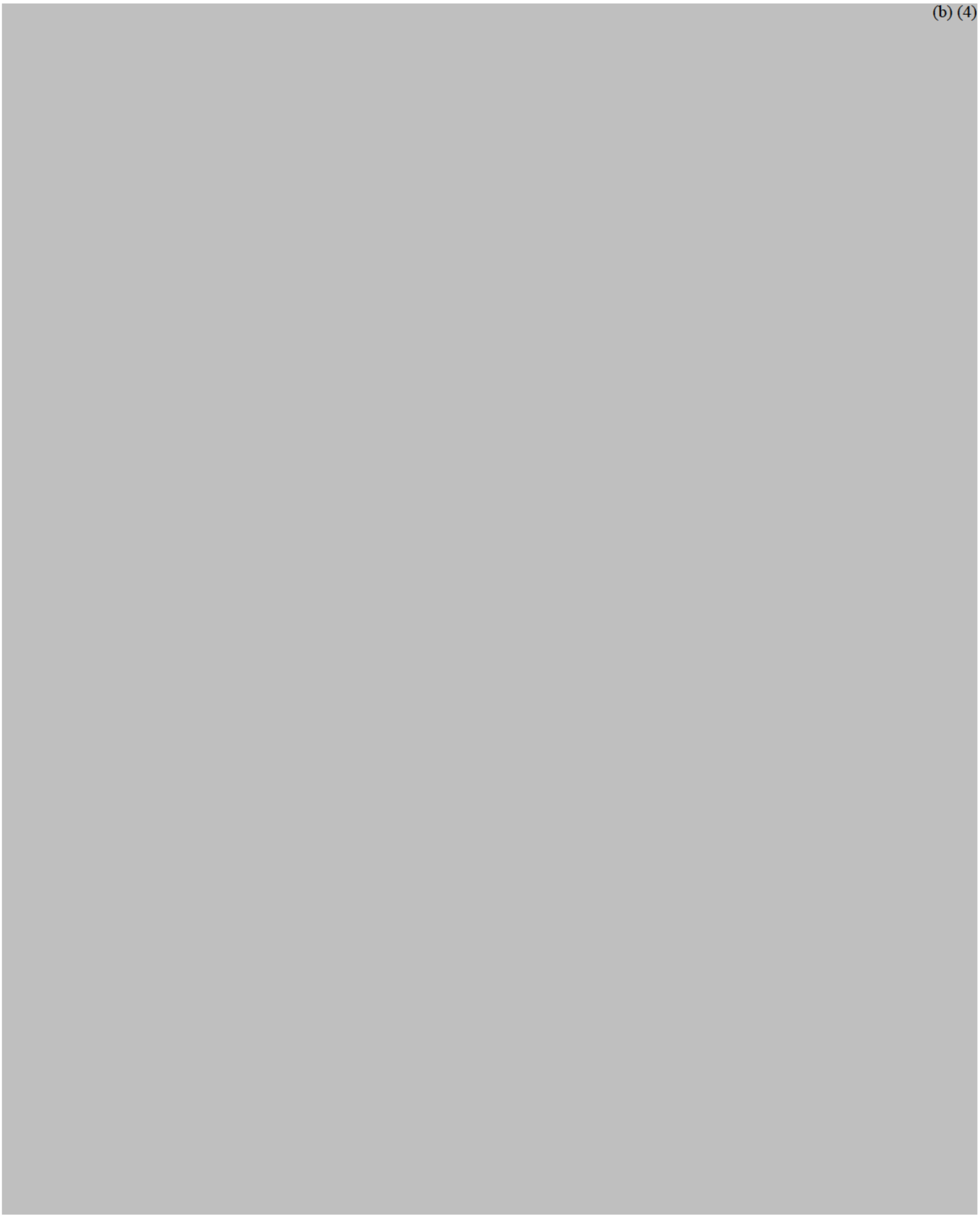

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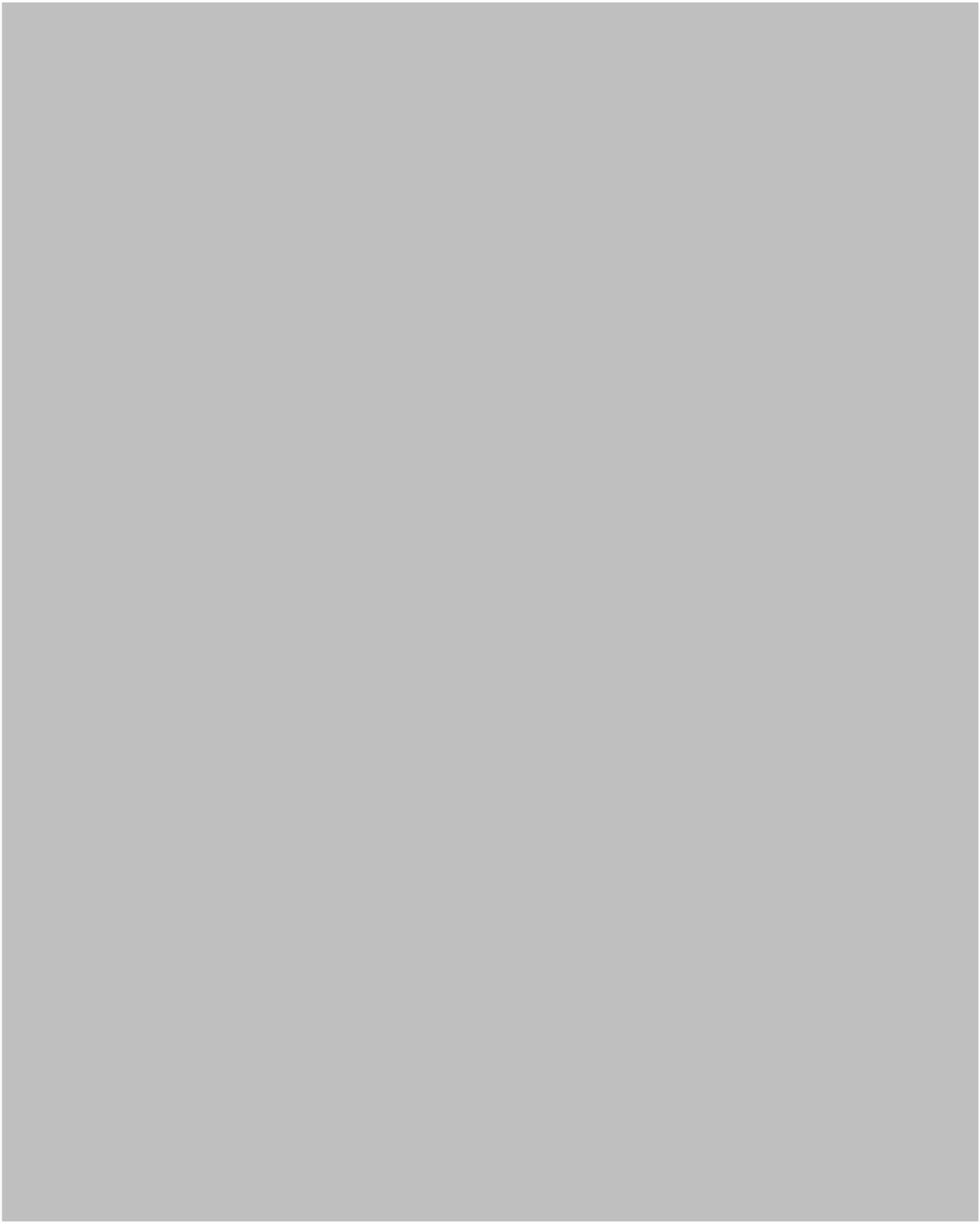

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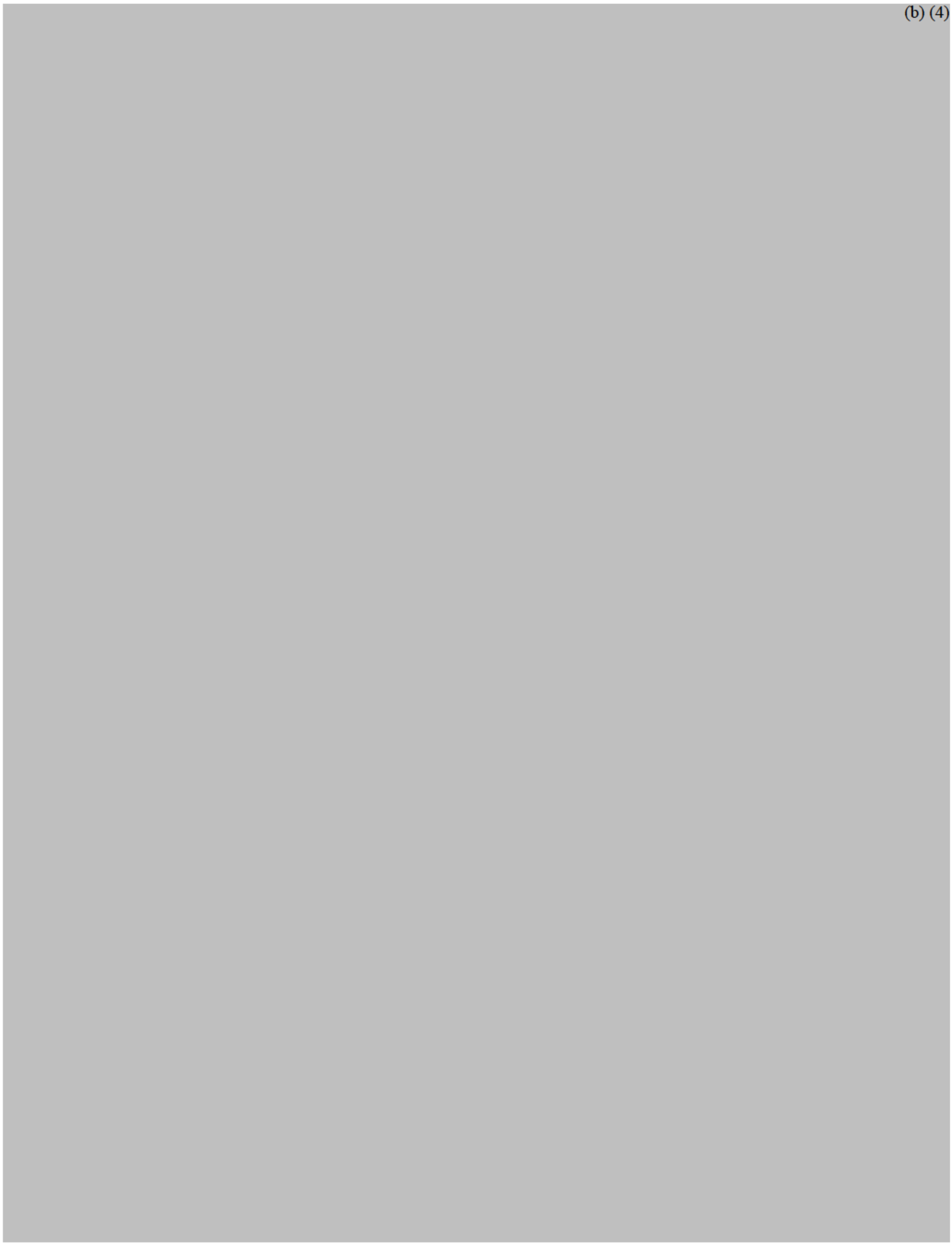

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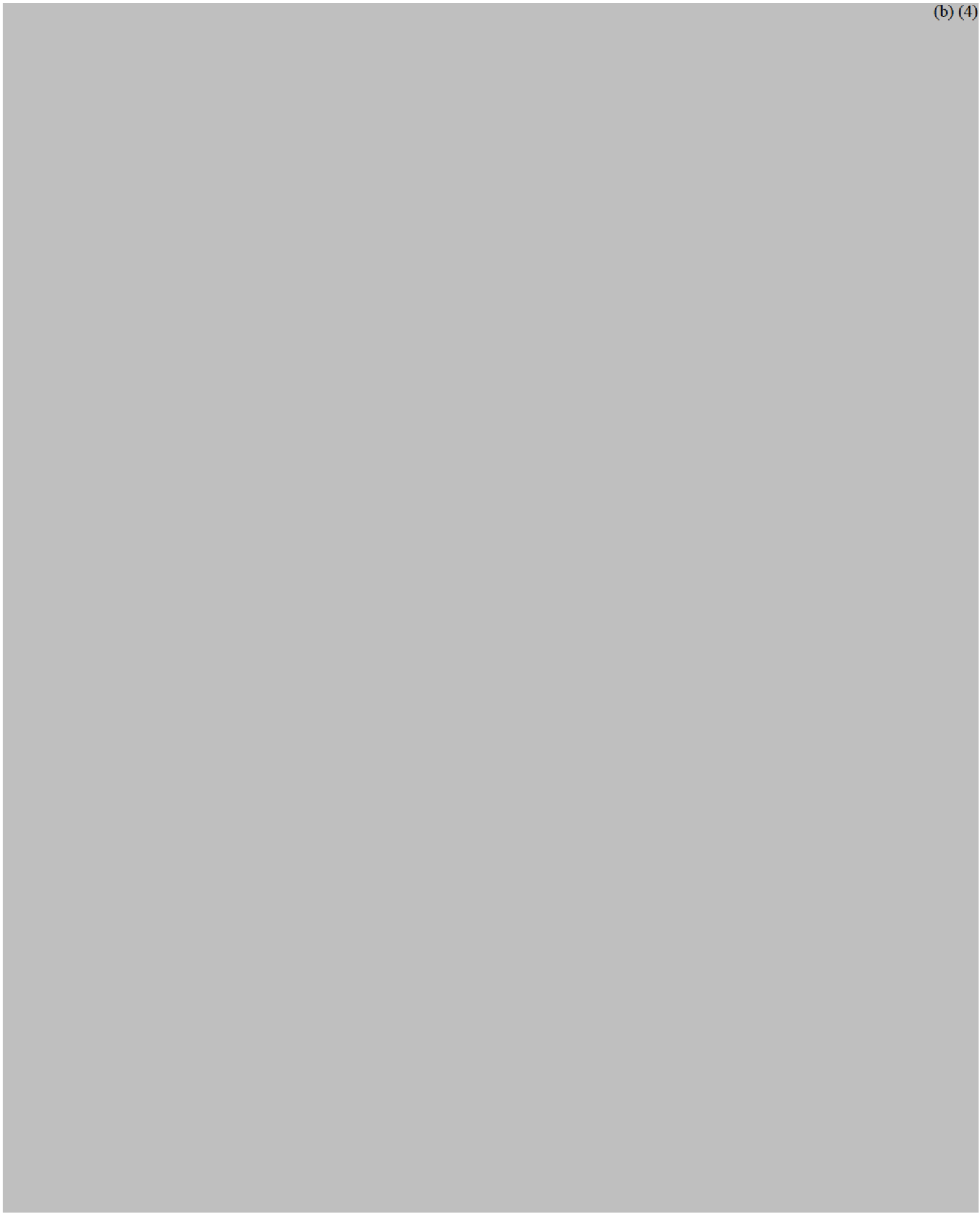

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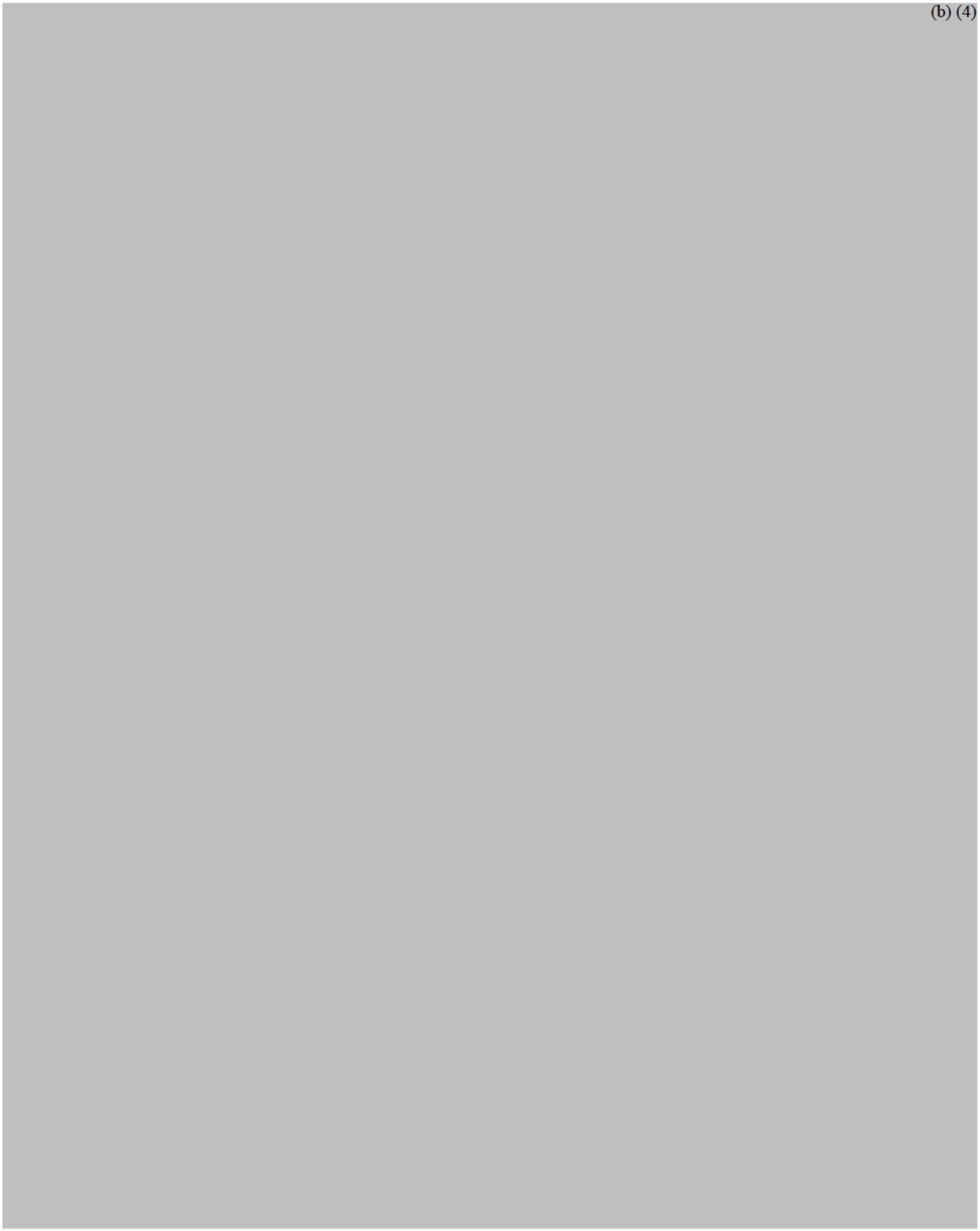

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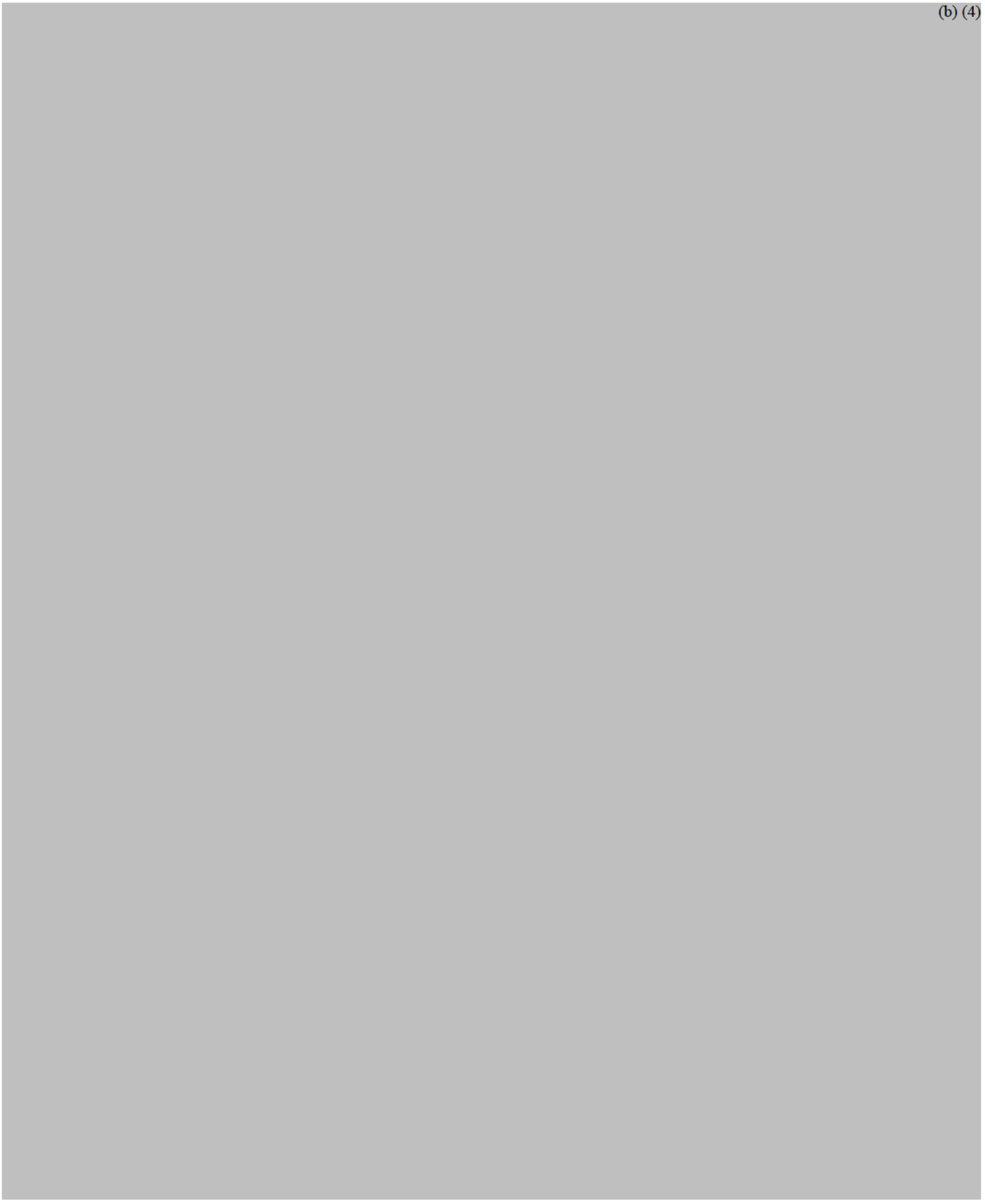

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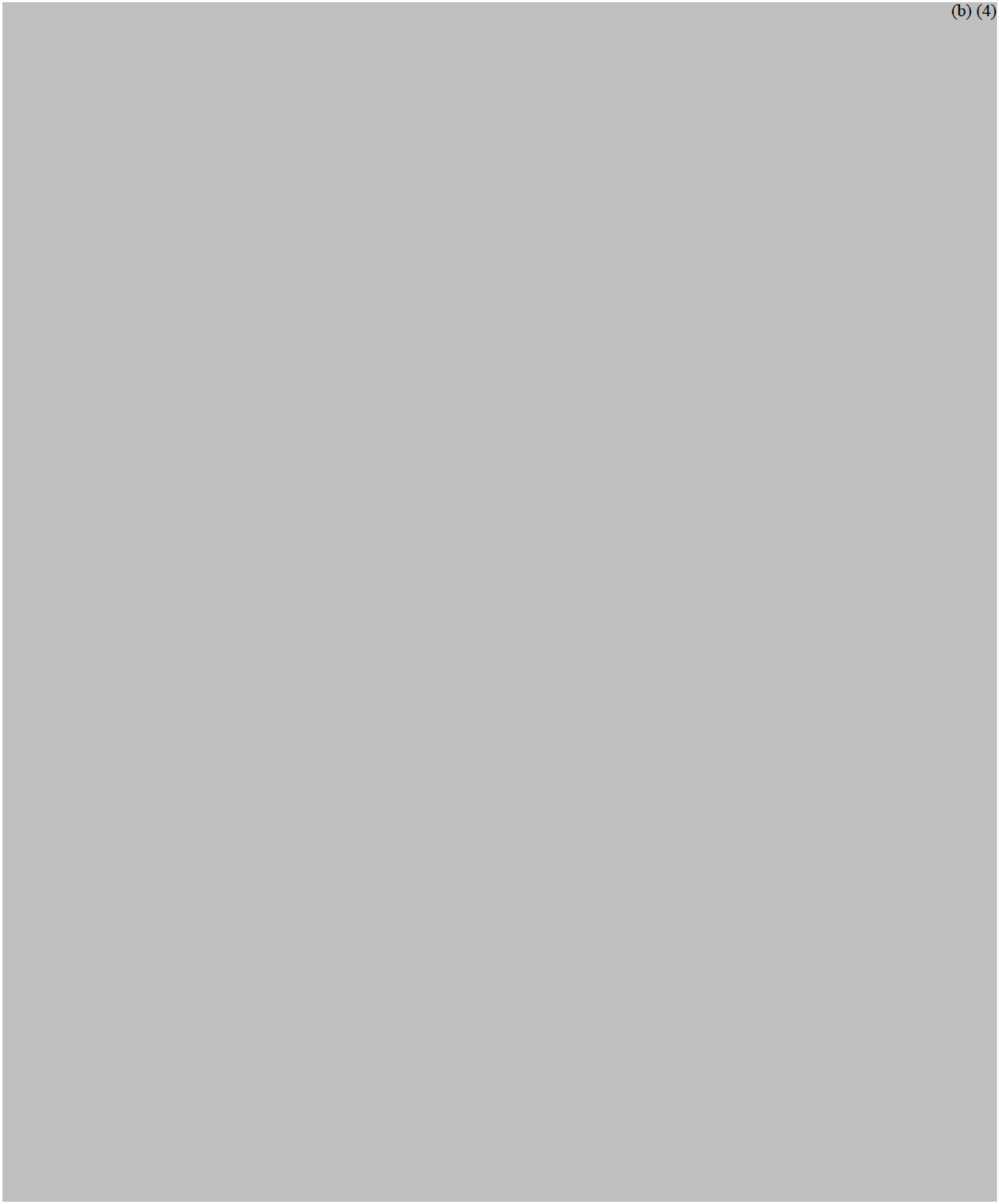

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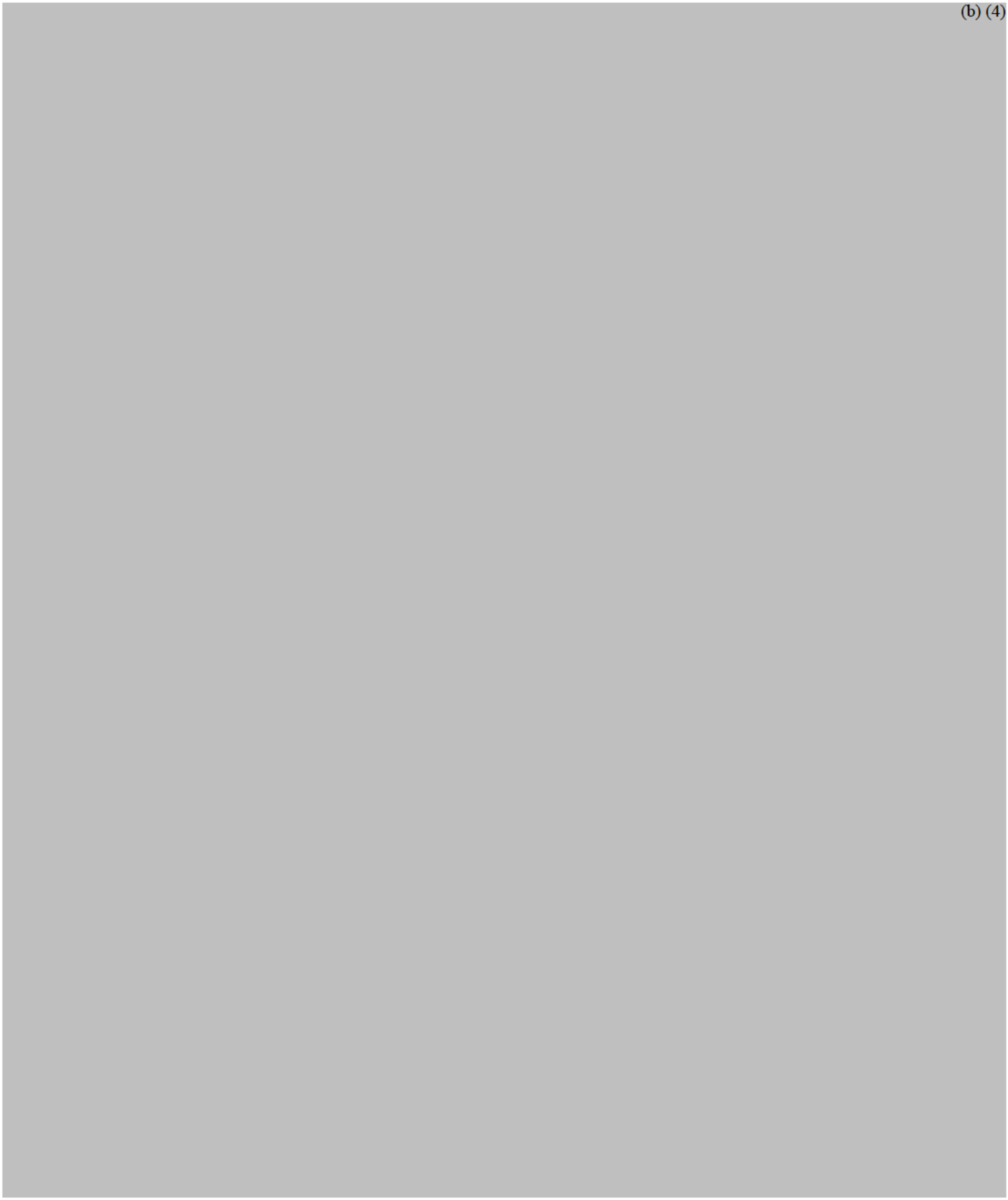

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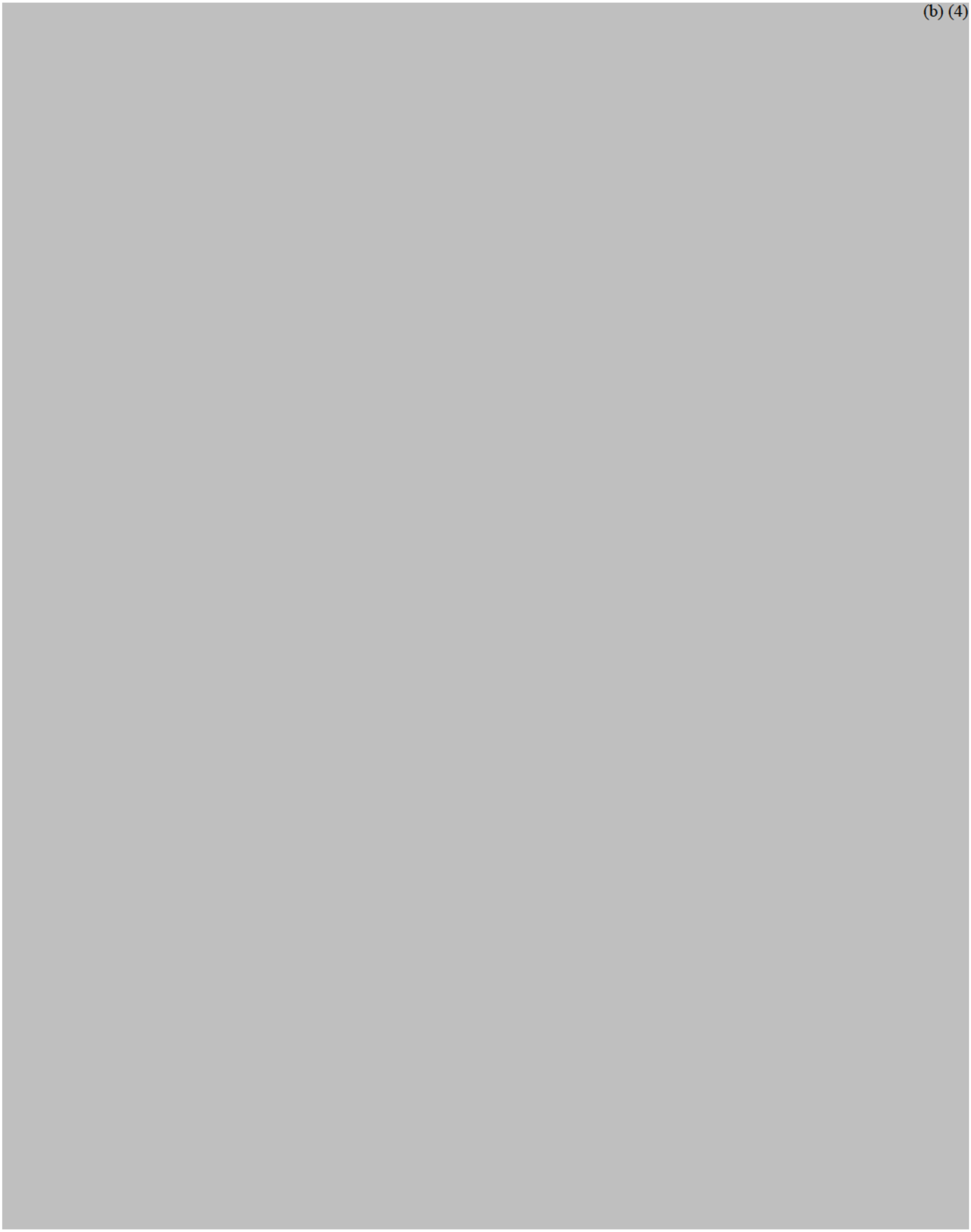

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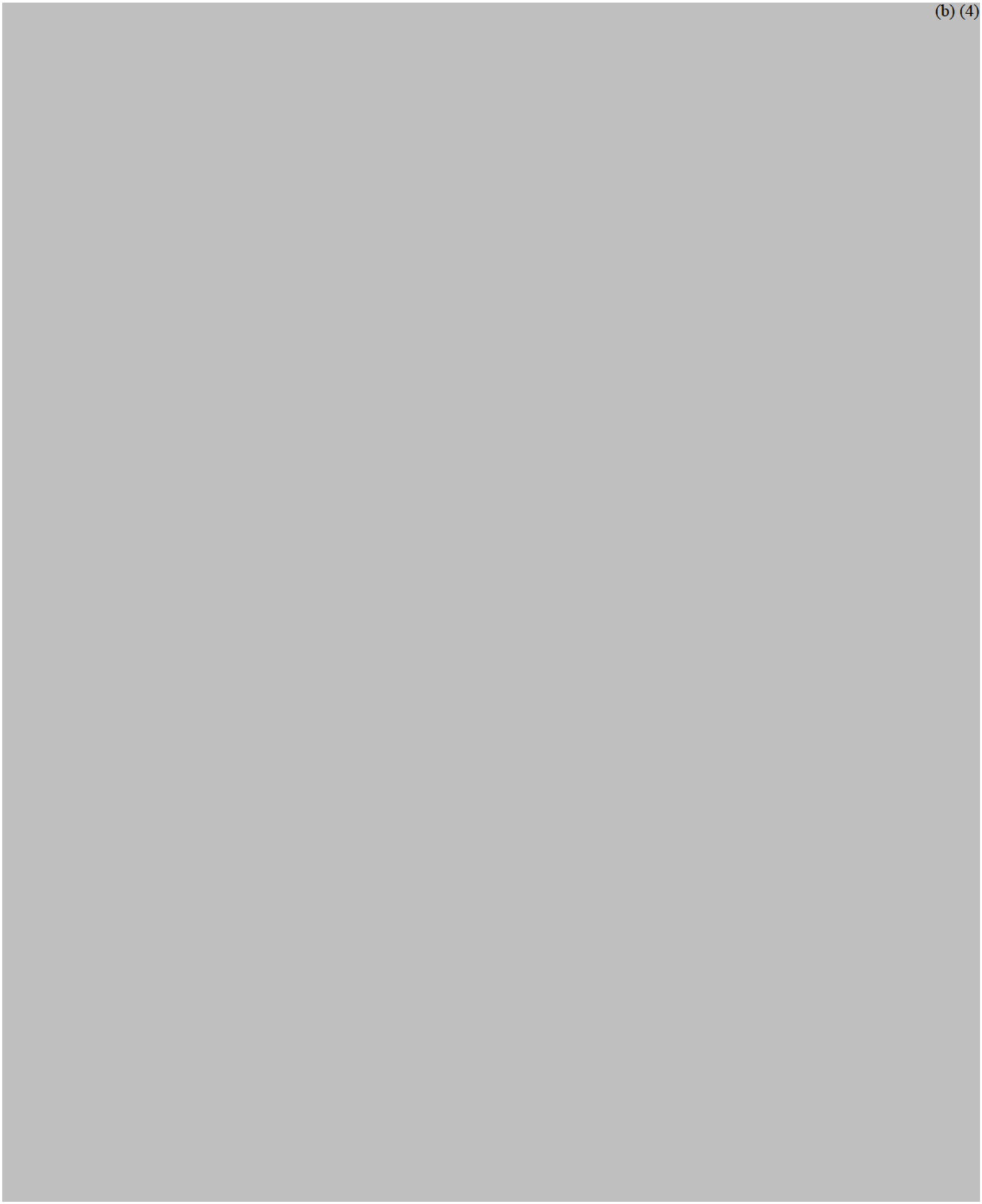

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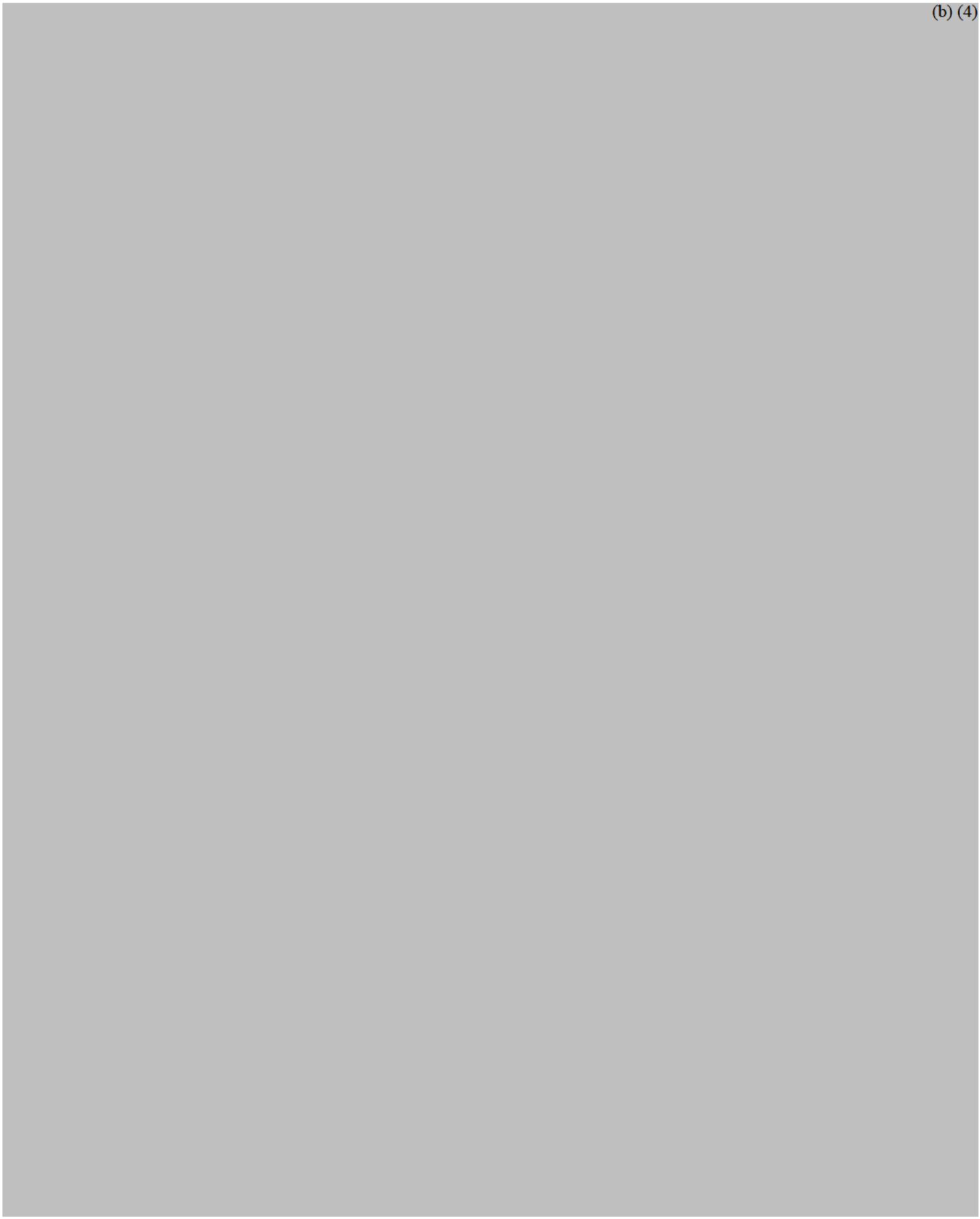

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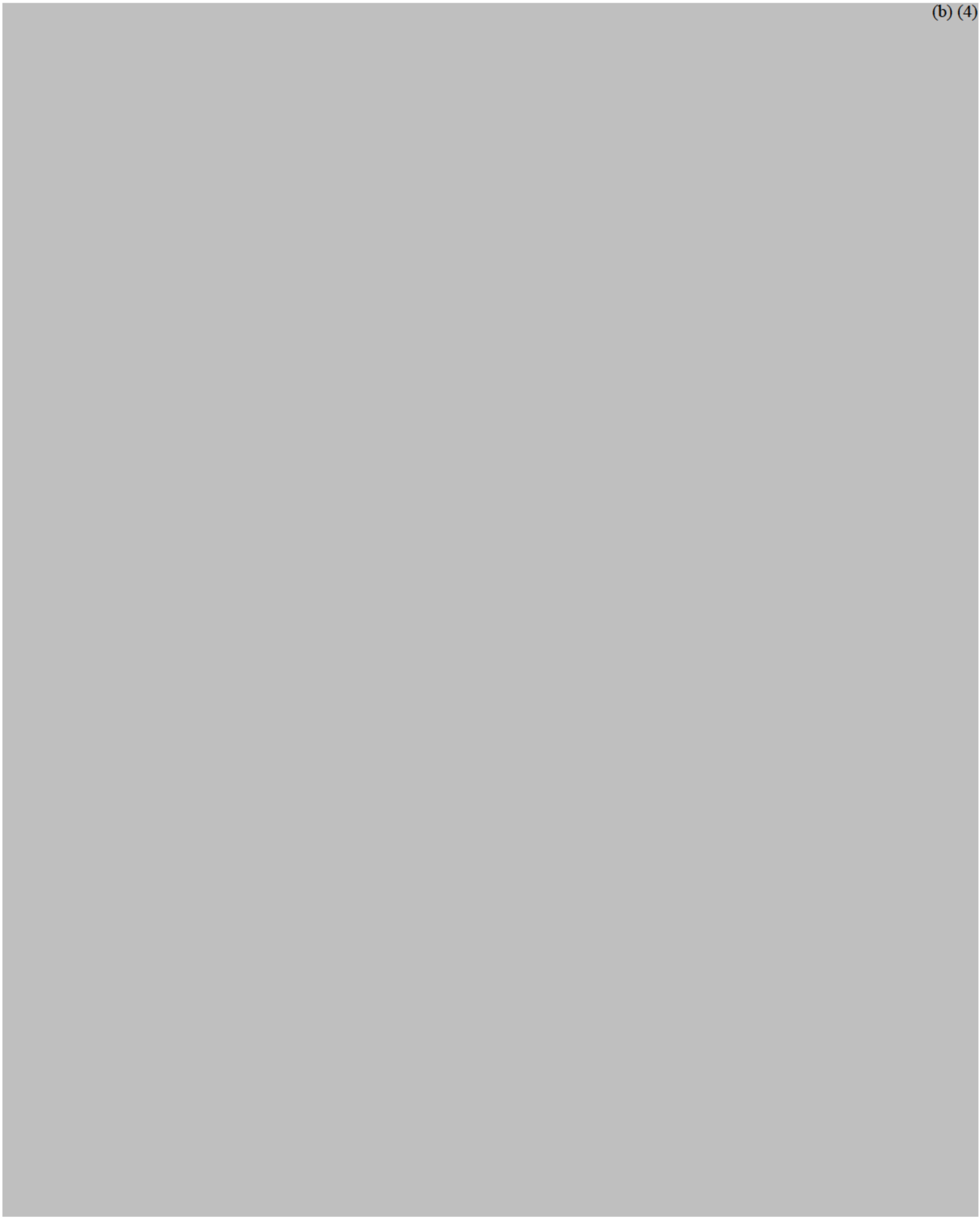

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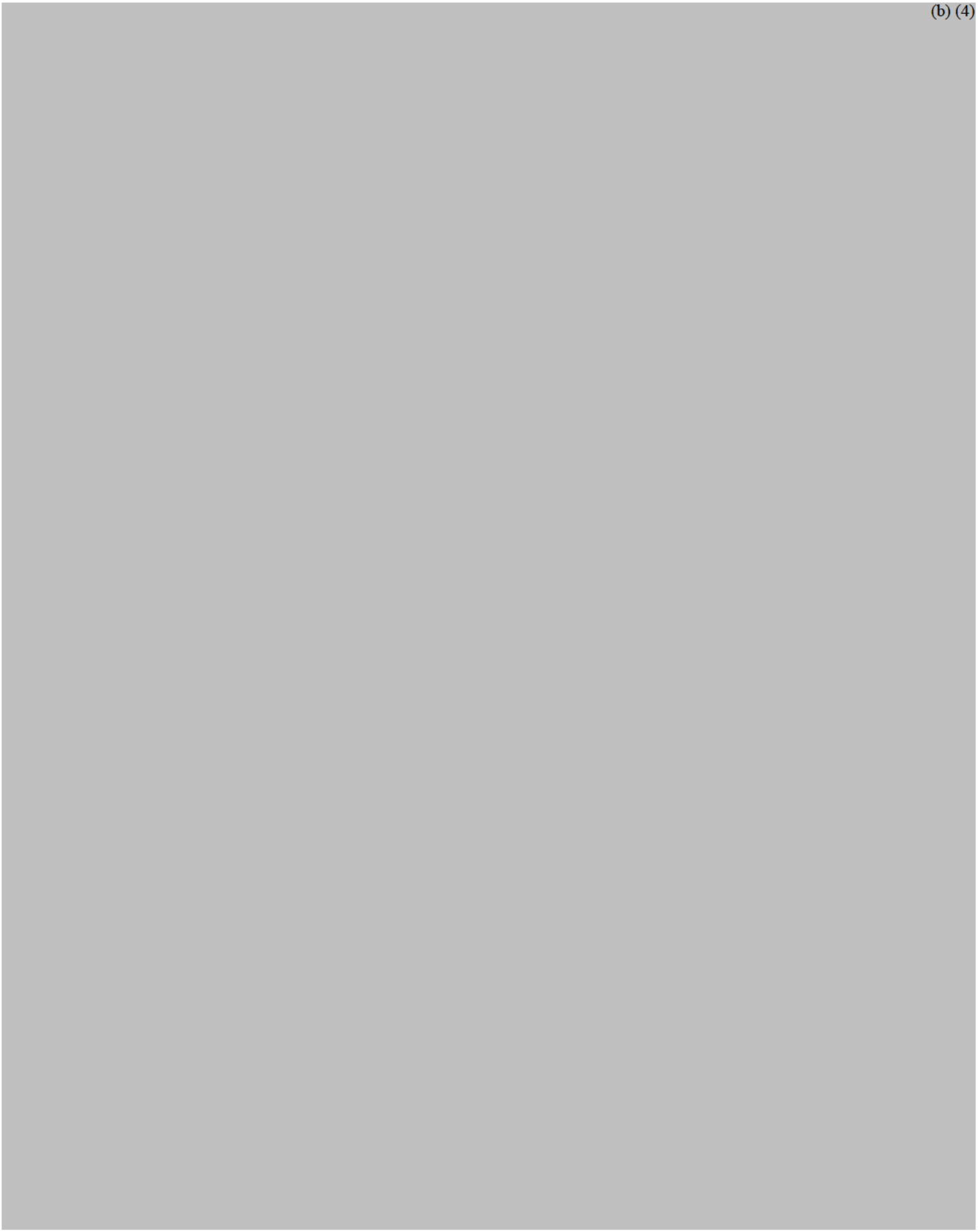

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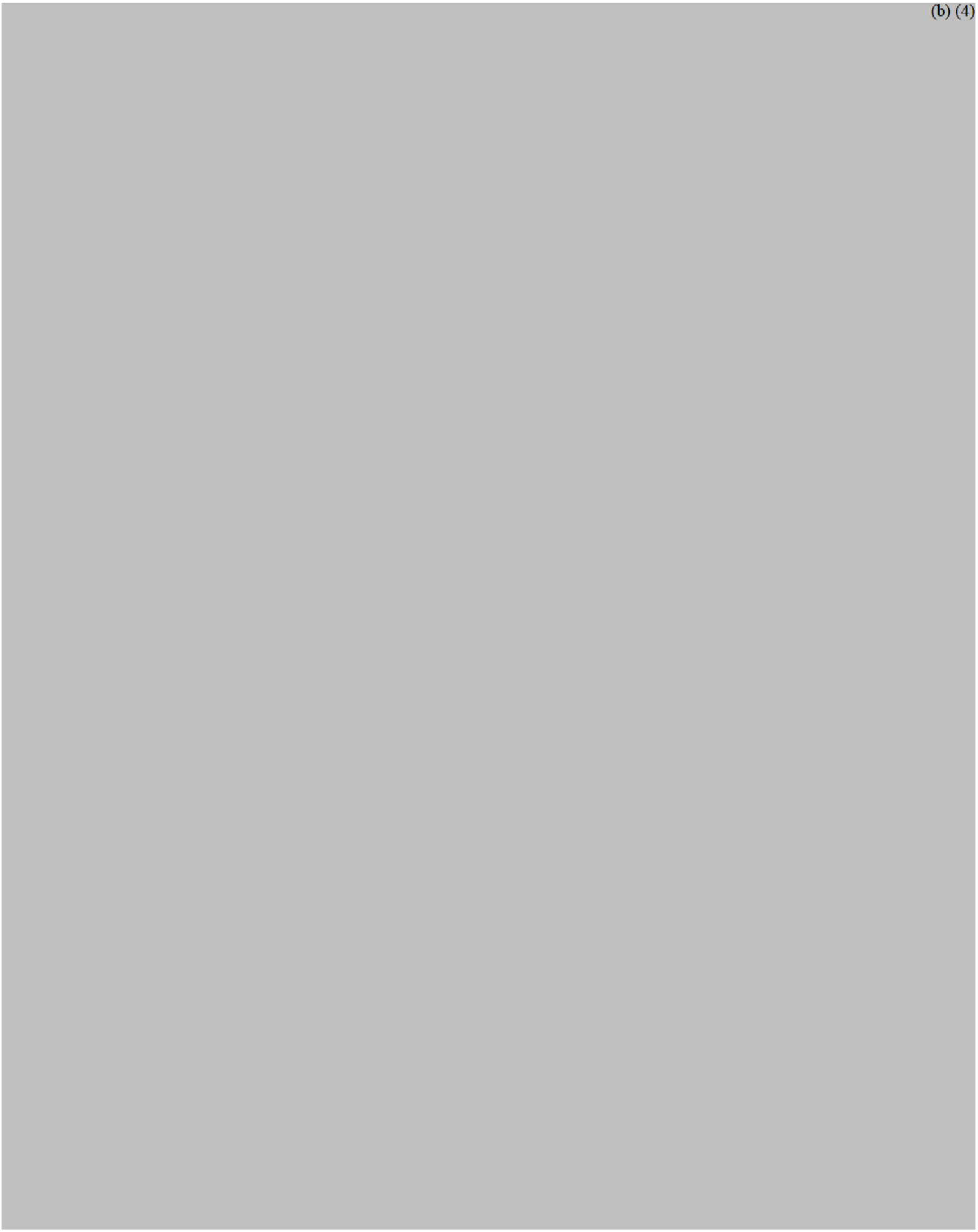

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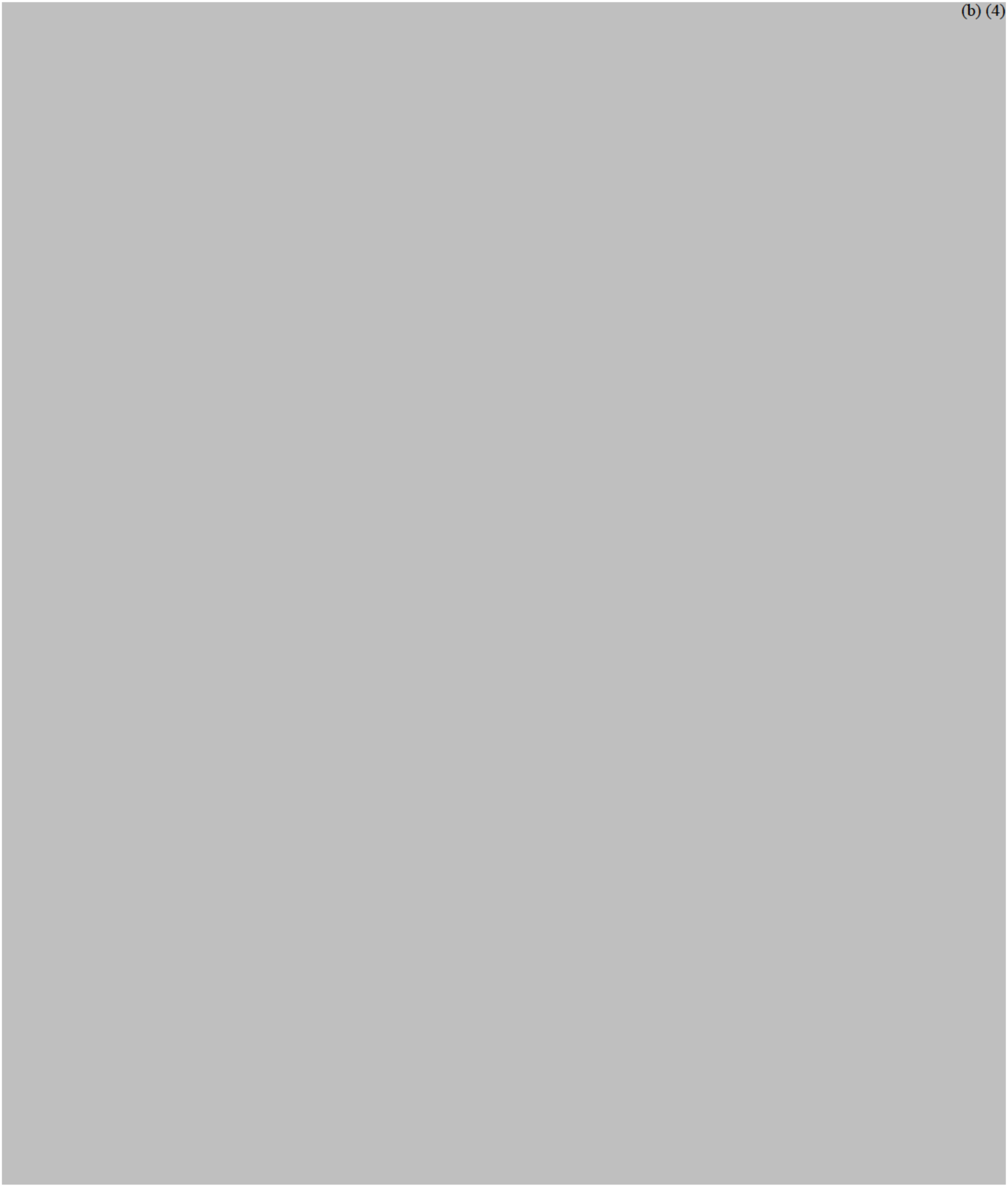

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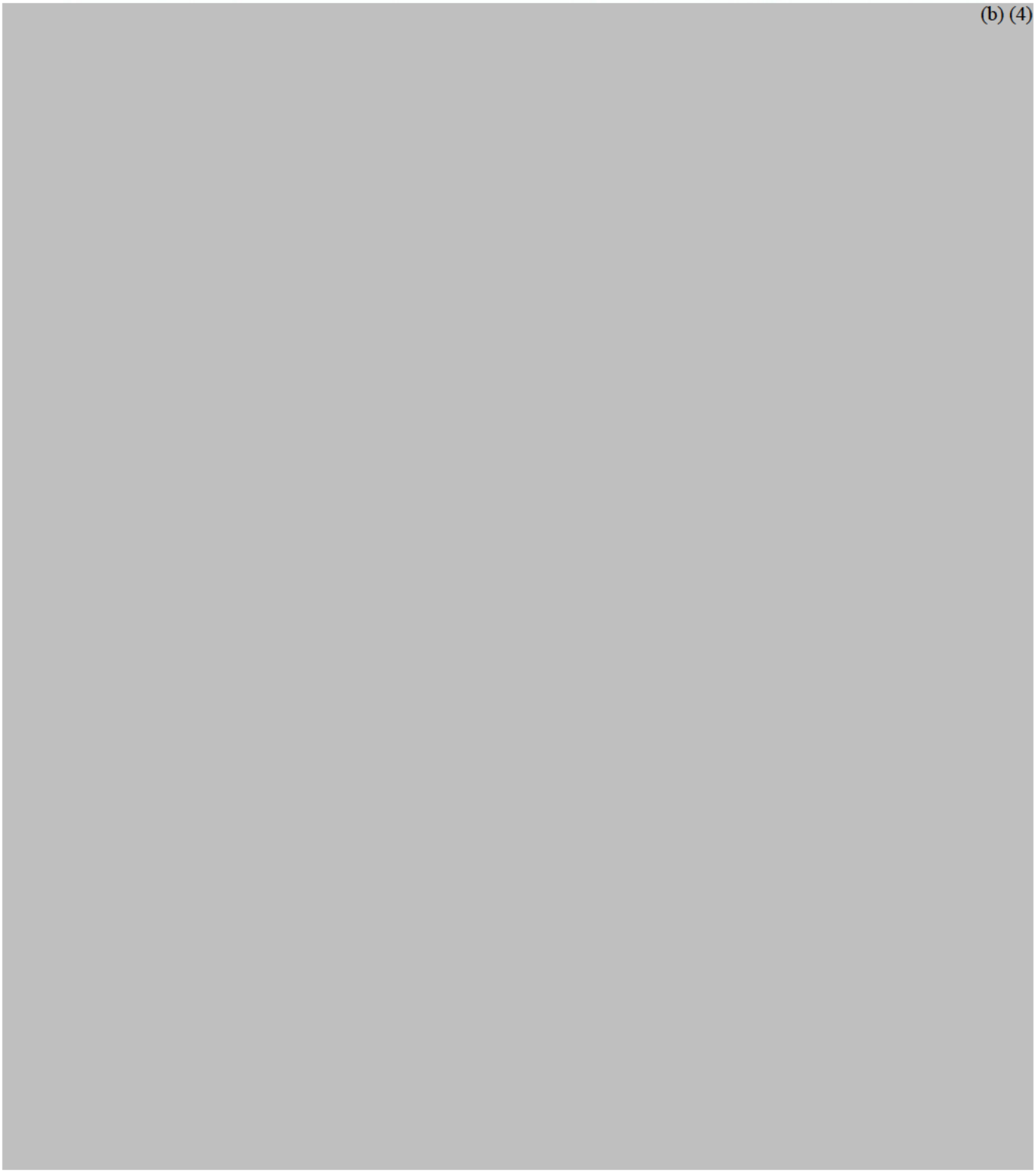
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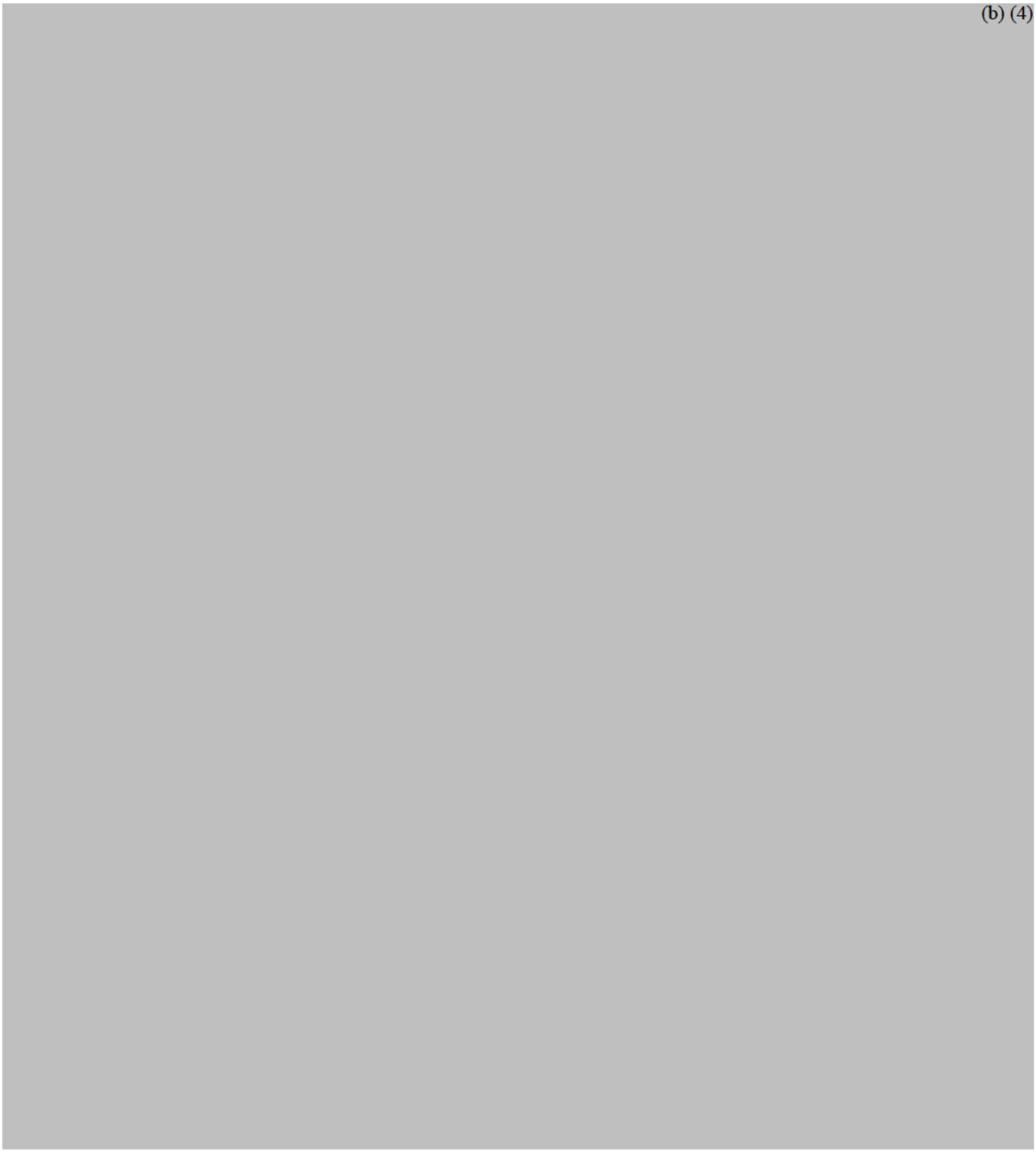

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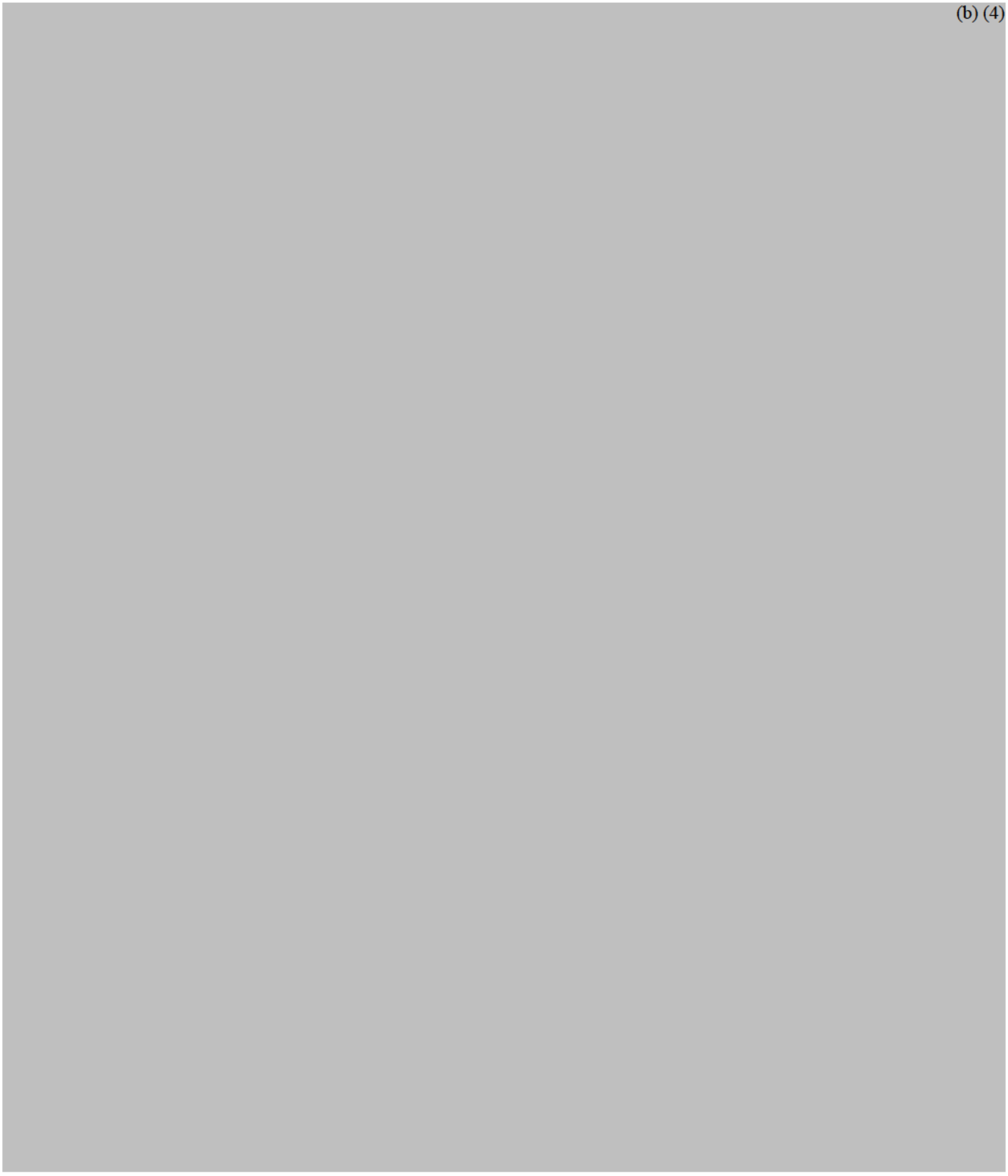

**D-2) The absence of viable cell in final product**

CJ L-methionine final product is tested by viable production cell test. CJ L-methionine solution did not show any production cell colony during incubation. This result indicated that there is no viable production cell in the test sample. Even though all the safety of production strain E.coli (b) (4) has been confirmed as above, CJ did not allow any inclusion of the production strain in the final product. After the fermentation, all the cells remaining in the solution is separated by two step separations. (b) (4)

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## Analysis Report

## TITLE: Detection of residual production strain in L-methionine

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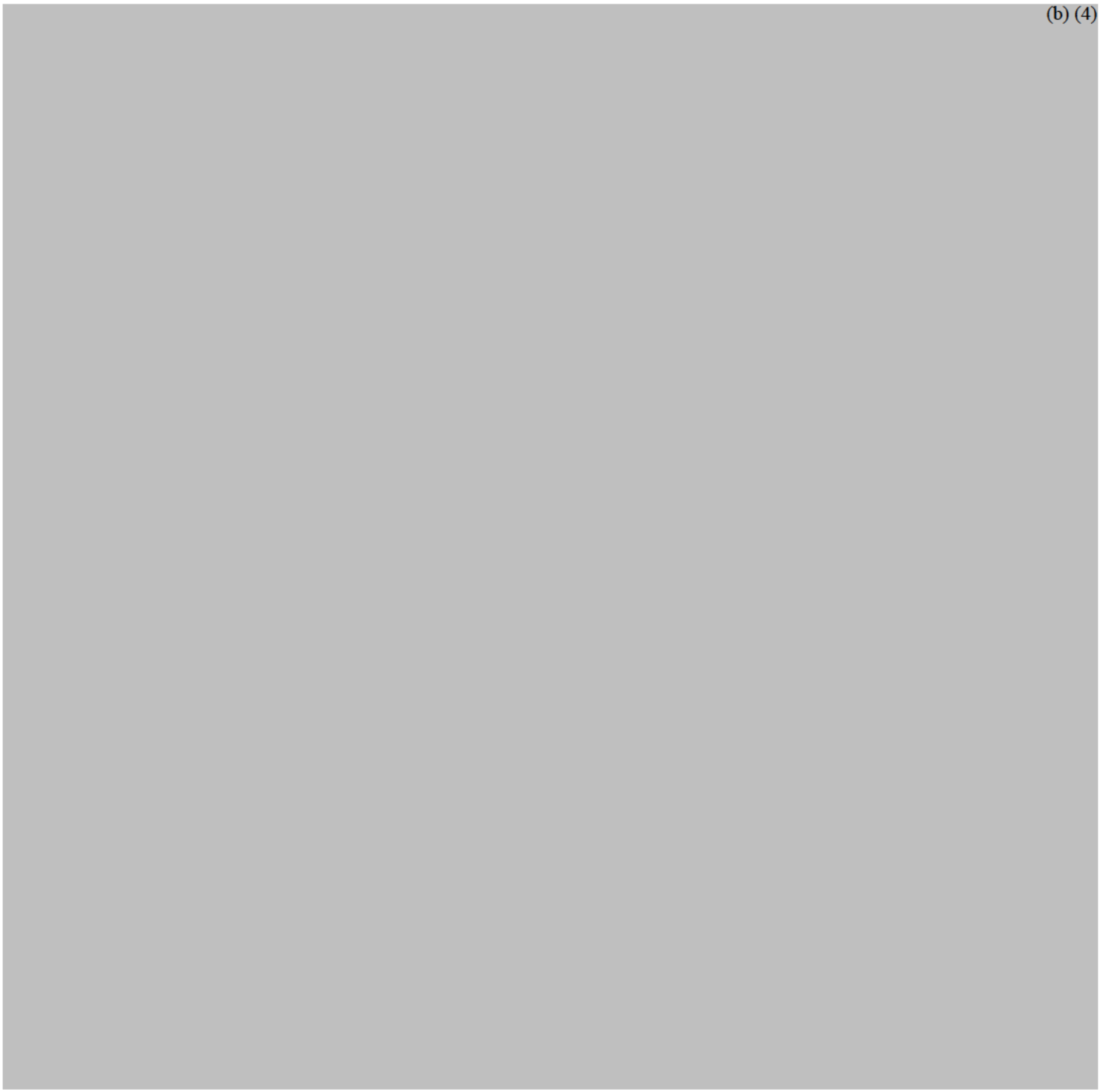

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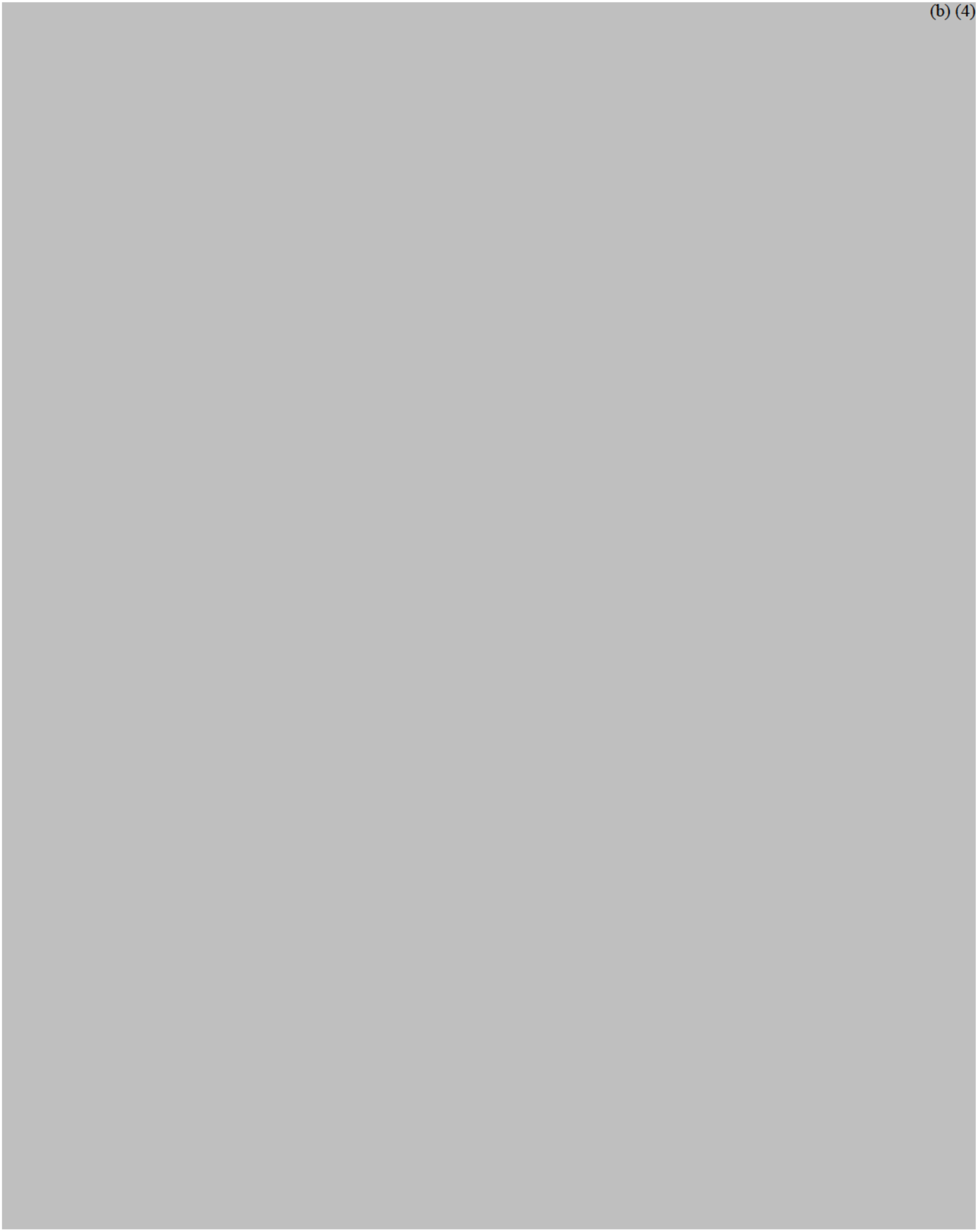

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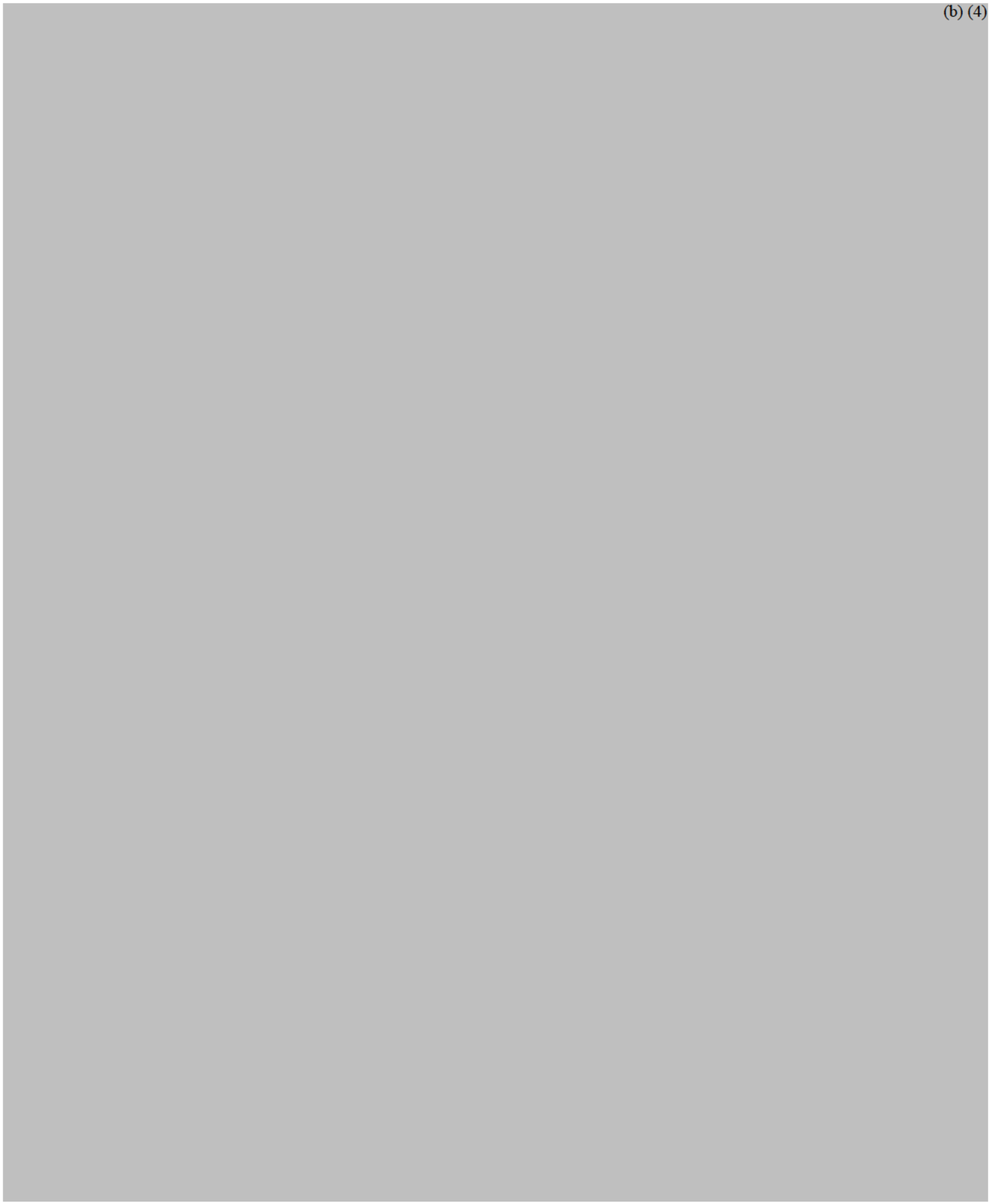

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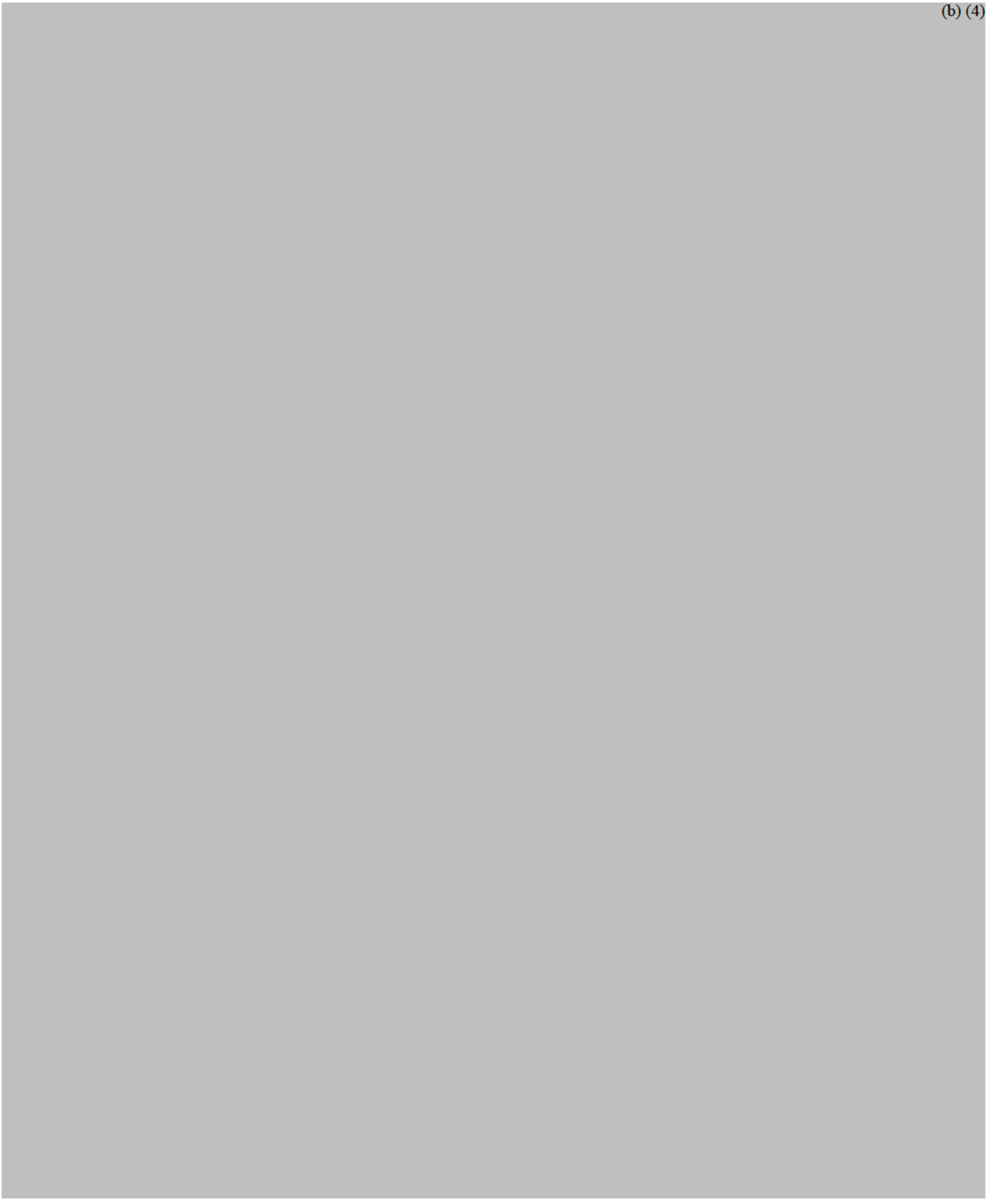

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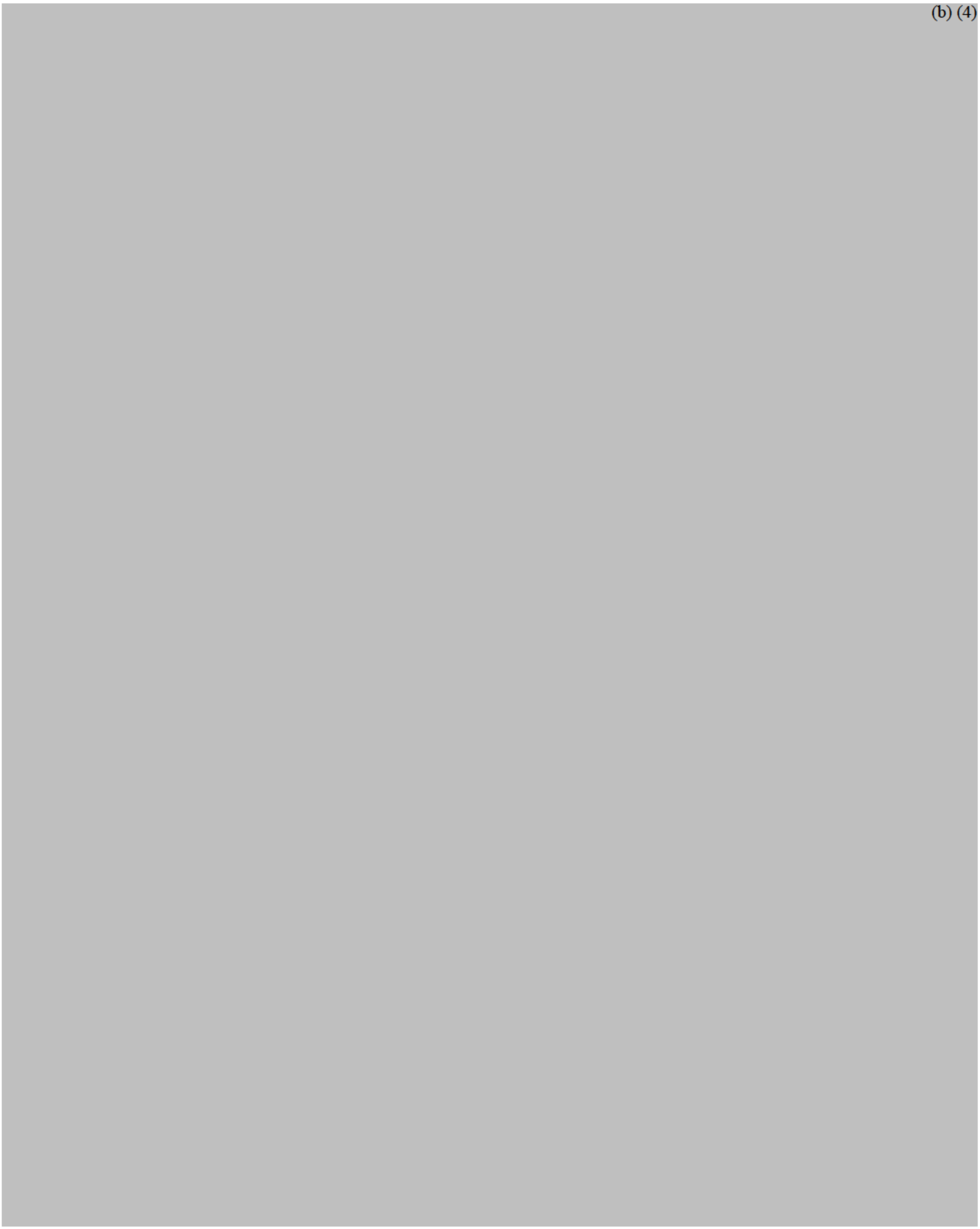

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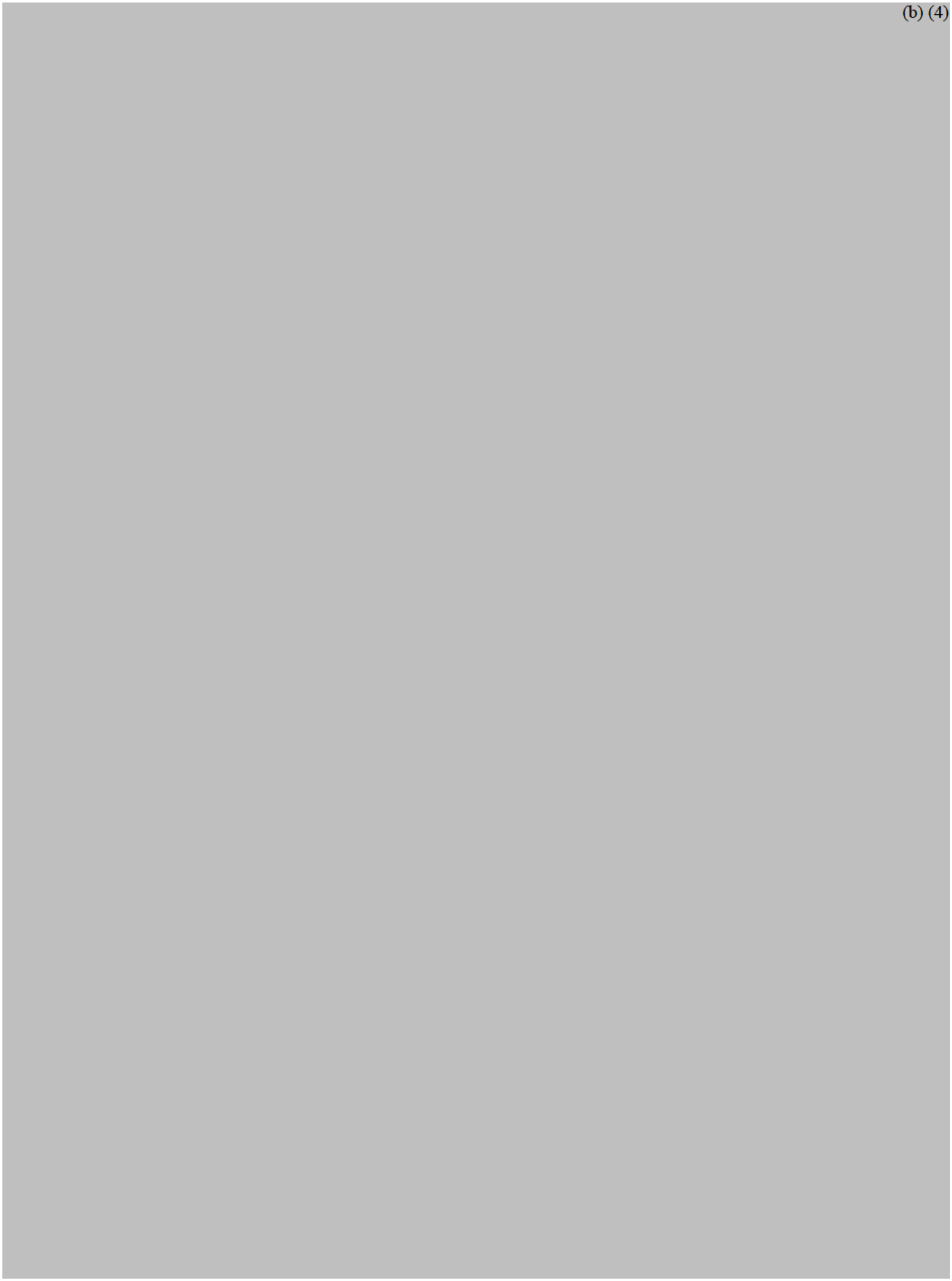

## D-3) The absence of residual enzyme in final product

CJ L-methionine was made by the enzymatic conversion (b) (4) (b) (4) and conversion enzyme was produced by the fermentation of E.coli (b) (4) (b) (4) (b) (4) From the analysis, there was no remaining enzyme activity in the final product. Hence, it was confirmed there is no remaining enzyme activity in the final product.

# REPORT

# The study about

# Conversion Enzyme remaining

# in the CJ L-Methionine

**< Confidential >**

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1. **TITLE**: The study about Conversion Enzyme remaining in the CJ L-Methionine

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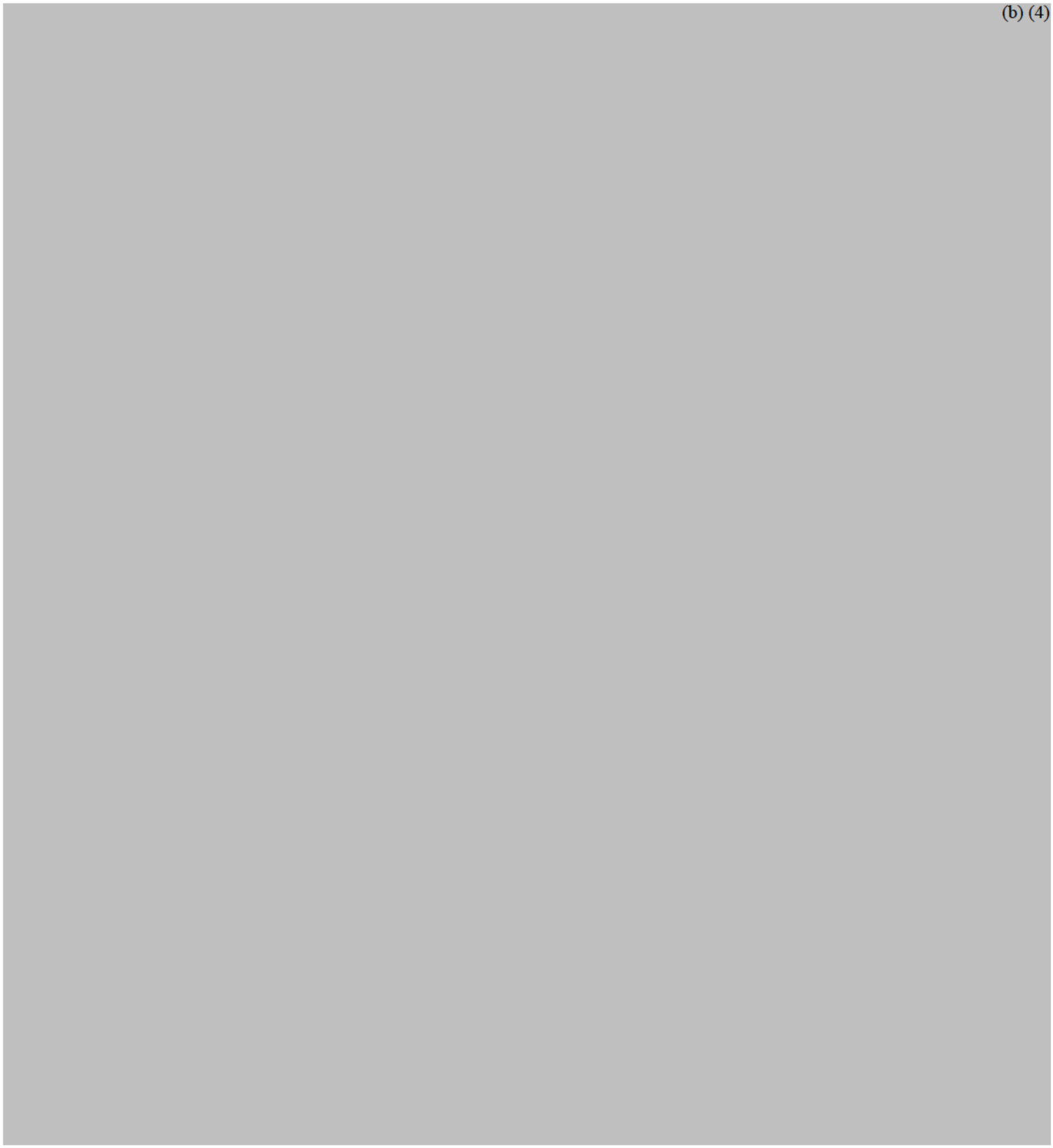

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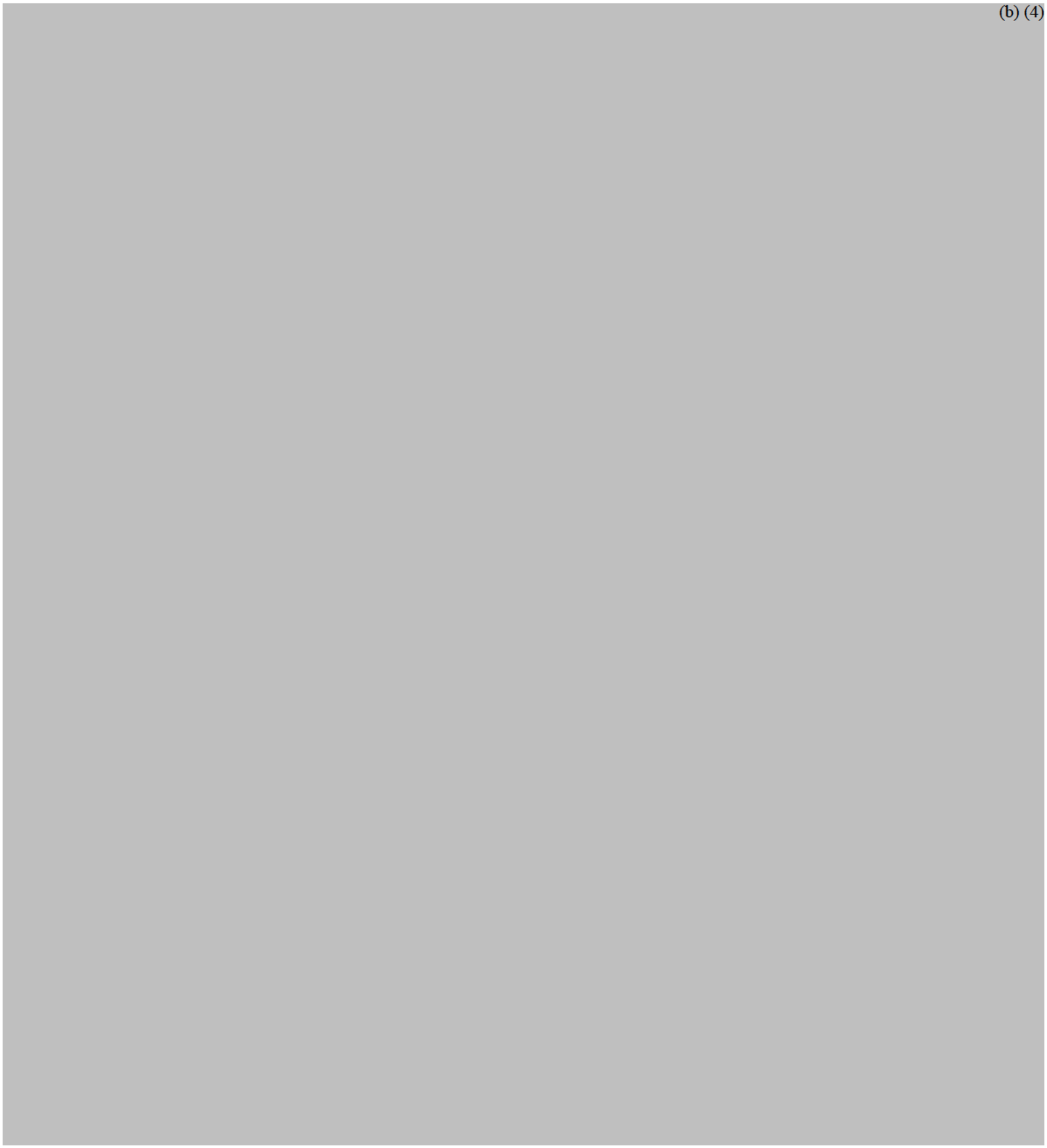

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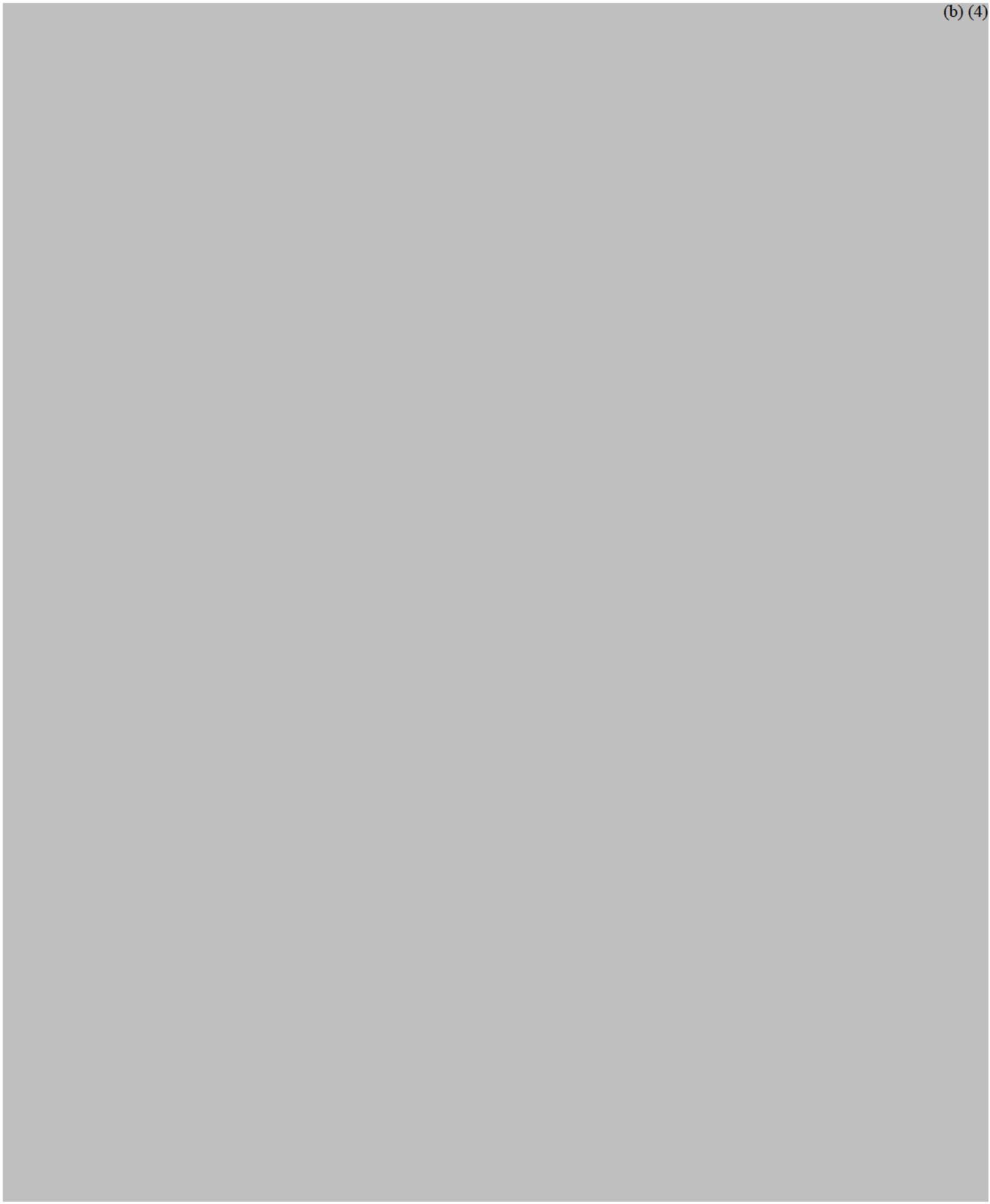

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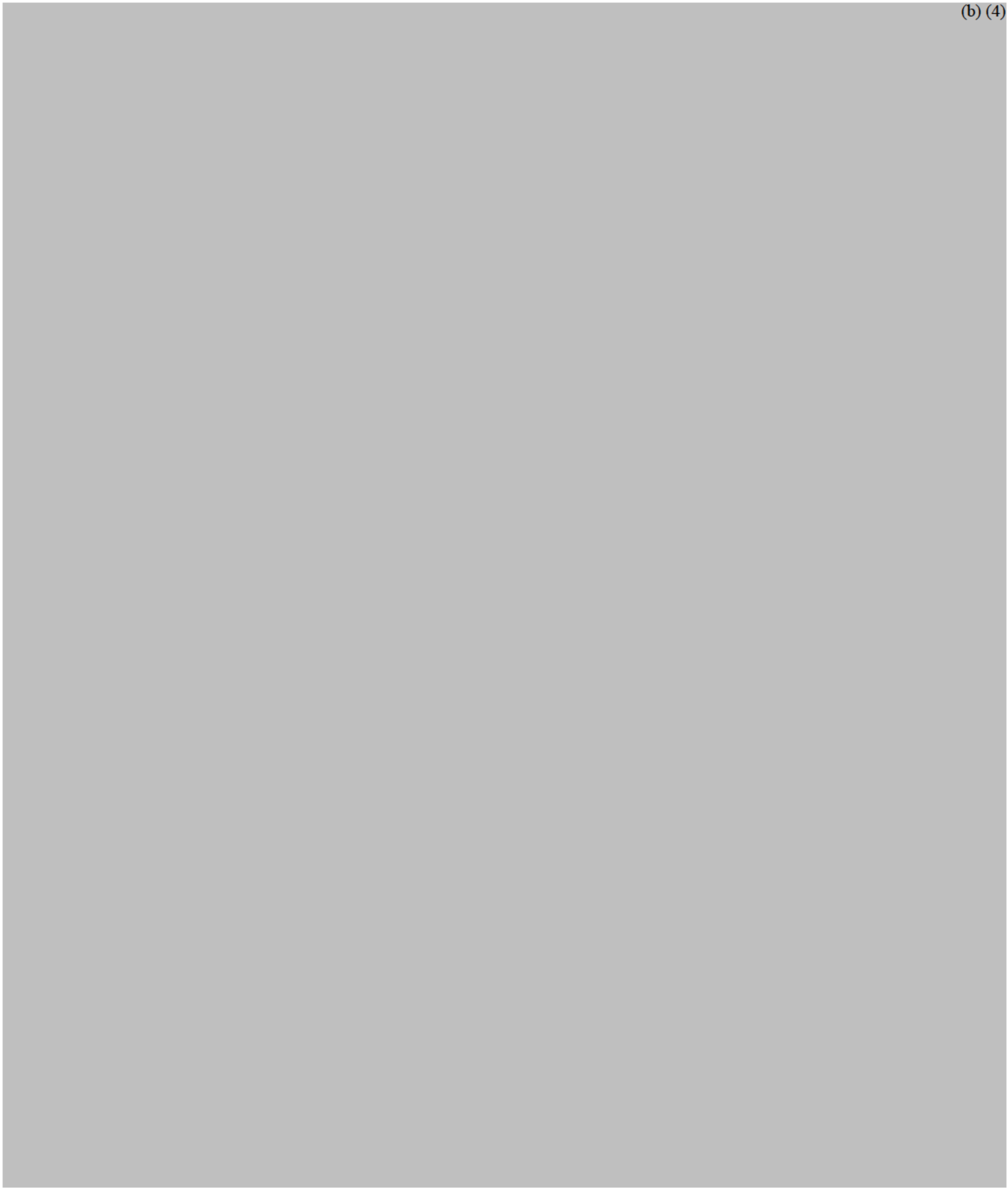

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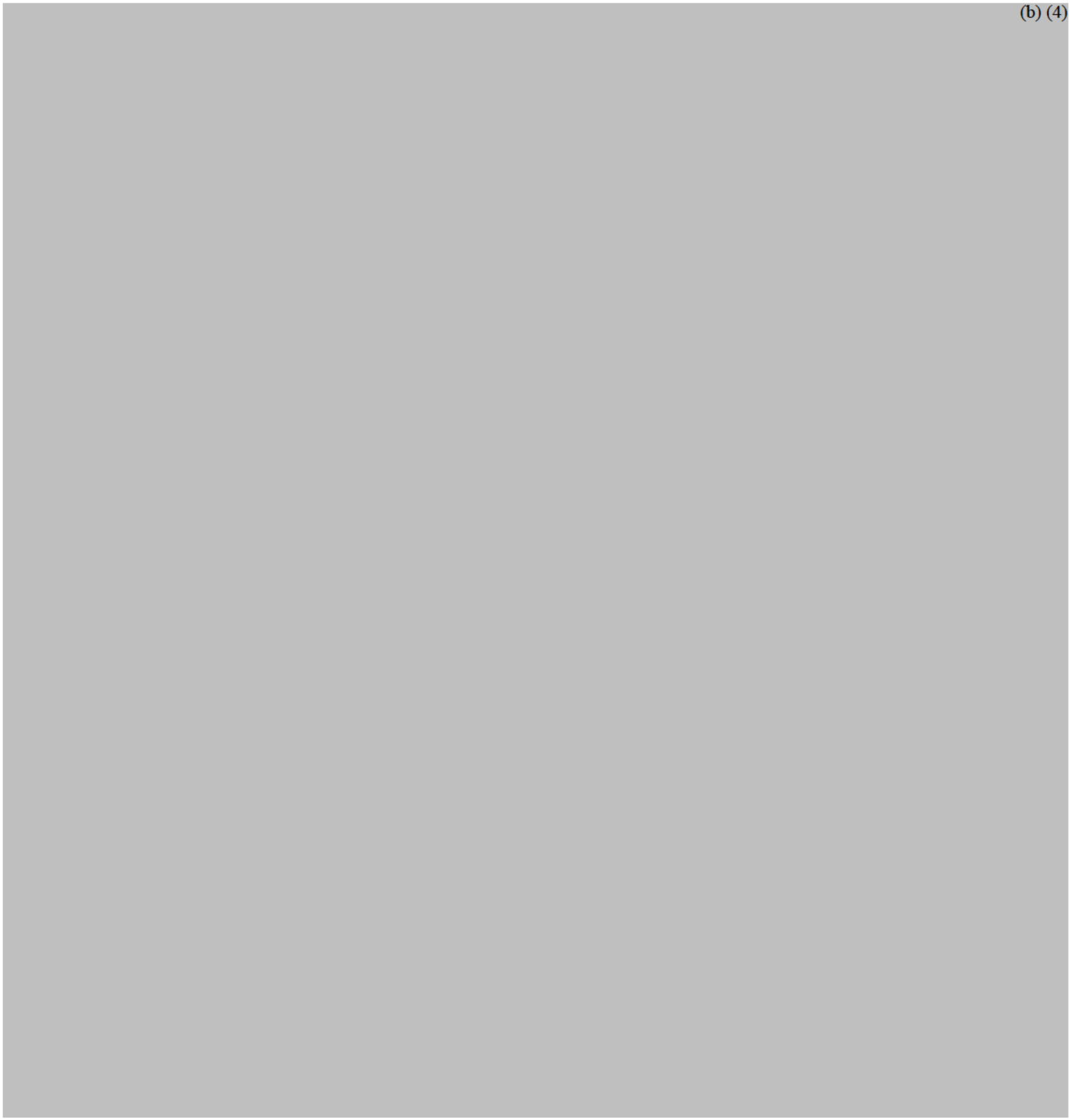

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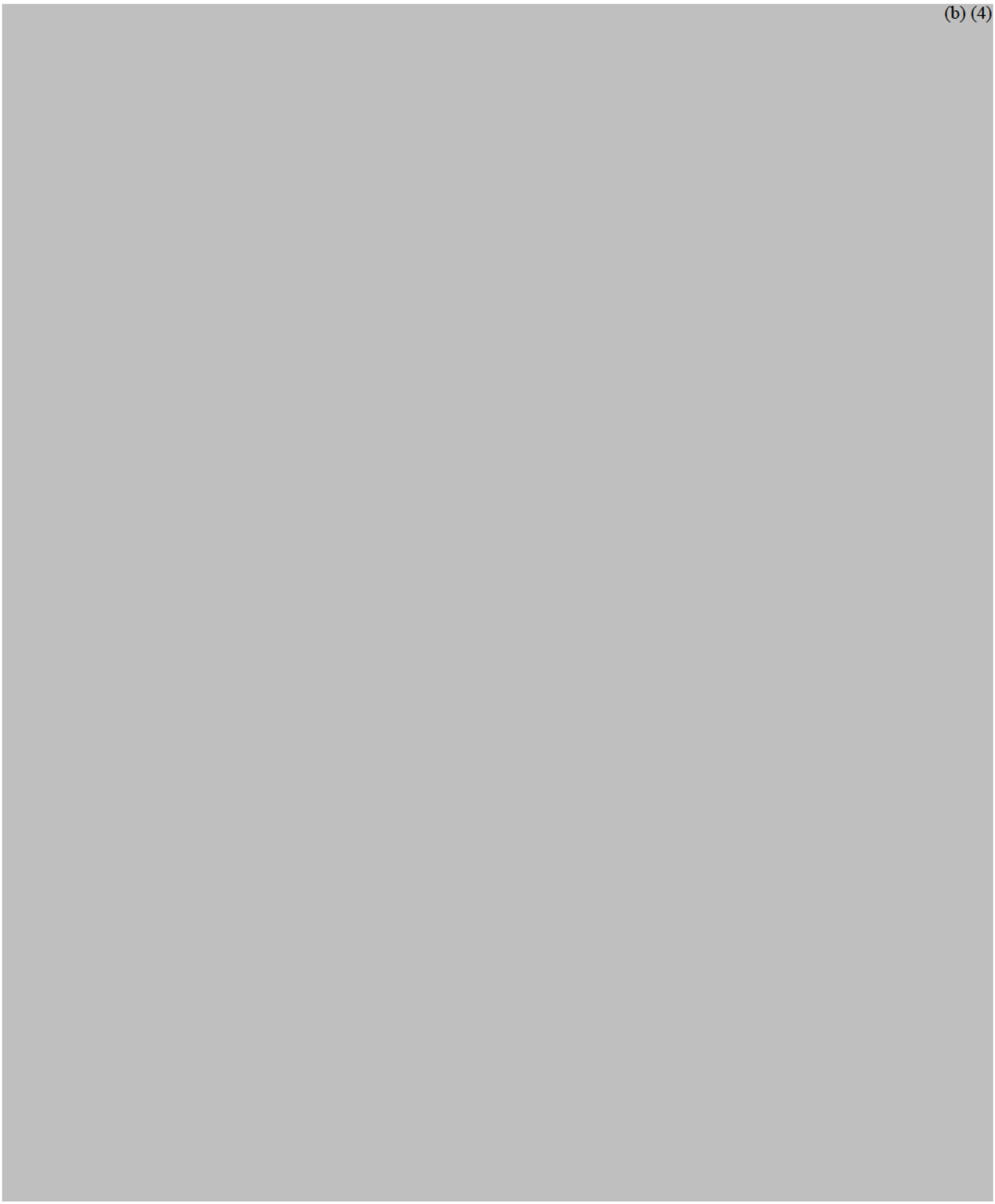

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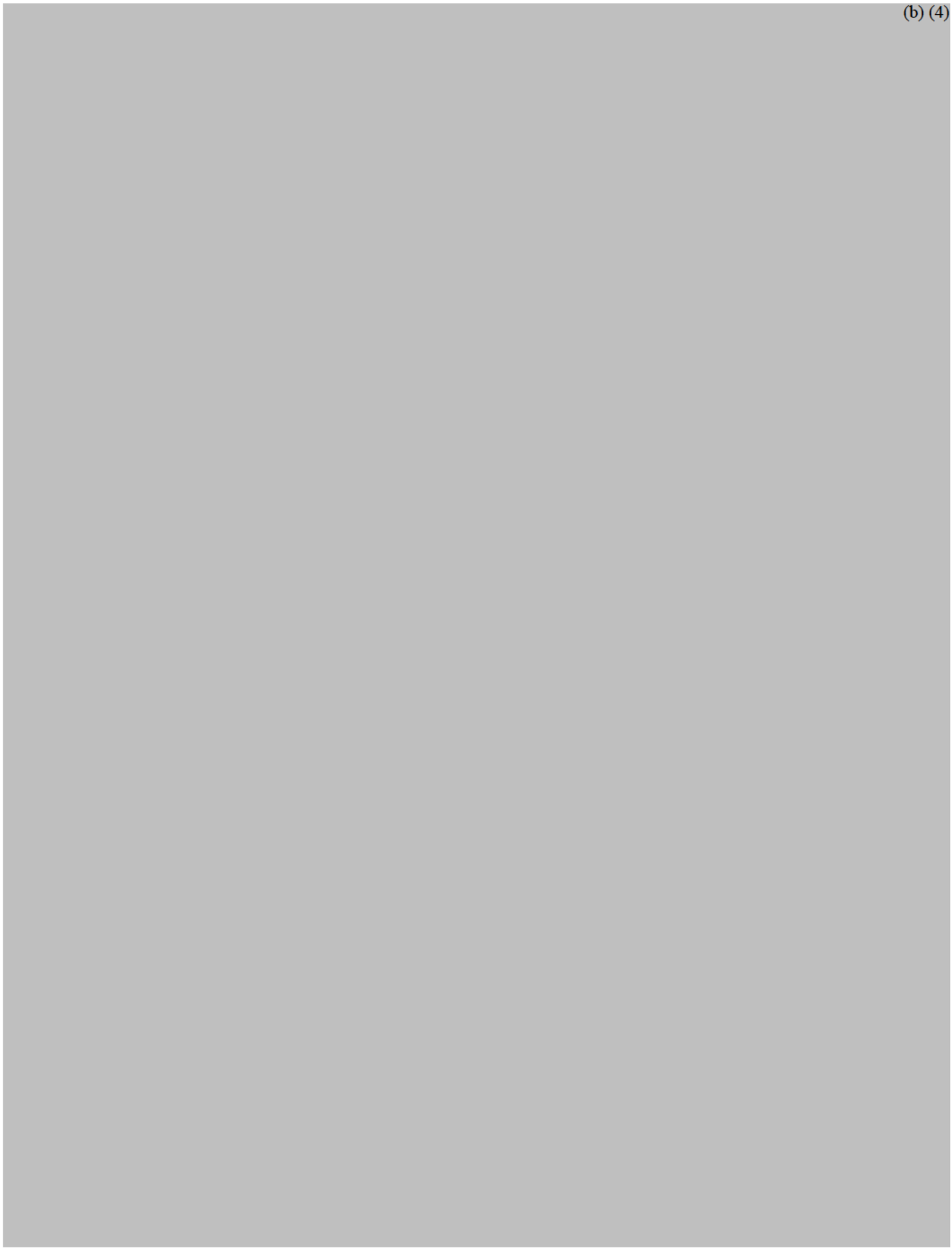

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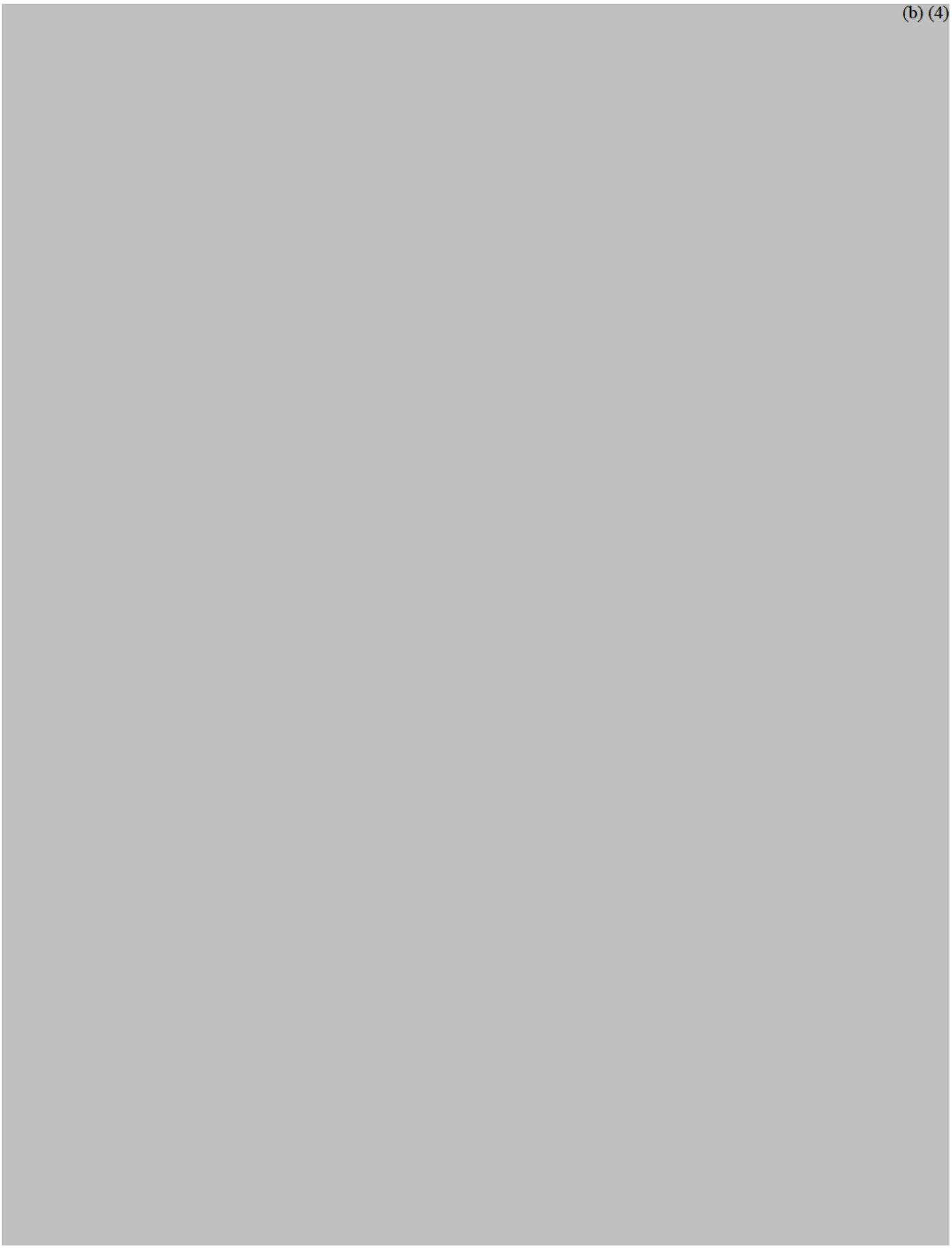

## E. Production strain safety

### E-1) Production strain form and safety

E. coli K12, as well as L-methionine production strain does not produce spores. Salmonella, Campylobacter, Escherichia, Shigella, and Vibrio species, and species from other genera can exist in a state where they are viable but cannot be cultured by normal microbiological methods. Studies show that E. coli K-12 strain in soil and water show that the E. coli cells were disappearing from the non-sterile microcosms studied. K-12 is recognized the non-pathogenic microorganisms and can't colonize in human intestinal tract. Because E. coli K-12 cell walls mucous membrane lack of adhesion recognition and other functions related ingredients. K-12 safety is certified by Europe and the USA and other countries safety evaluation institutions.

### E-2)Production strain factors test

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(b) (4) In addition, several control strains of E. coli, Shigella, and Salmonella were tested for comparison. With regard to the possible presence of known pathogenicity genes, Real-Time-PCR study was performed on the E. coli (b) (4) to detect the genes encoding the virulence and pathogenicity factors. The result is that none of the genes of the tested virulence and pathogenicity factors were found to be present in the strains E. coli (b) (4). It was concluded that strains of E. coli (b) (4) do not possess any of the tested genes for pathogenicity factors.

Absence of the pathogenicity genes tested fits the demonstrated E. coli K12 ancestry. Strain E. coli K12 is universally reputed non-pathogenic. Strain E. coli K12 is not able to colonize the human intestinal tract under normal conditions, even after ingestion of billions of organisms. As noted above, K-12 is defective in cell wall components relevant to the ability to recognize and adhere to the mucosal surface of colonic cells. The normal flora in residence in the colon thus can easily exclude K-12, and prevent it from colonizing the human colon. Based on the results of the studies on E. coli (b) (4) (b) (4), and body of evidence for the safety of E. coli K-12, the absence of pathogenicity of the E. coli (b) (4) strain is considered to be sufficiently proven.

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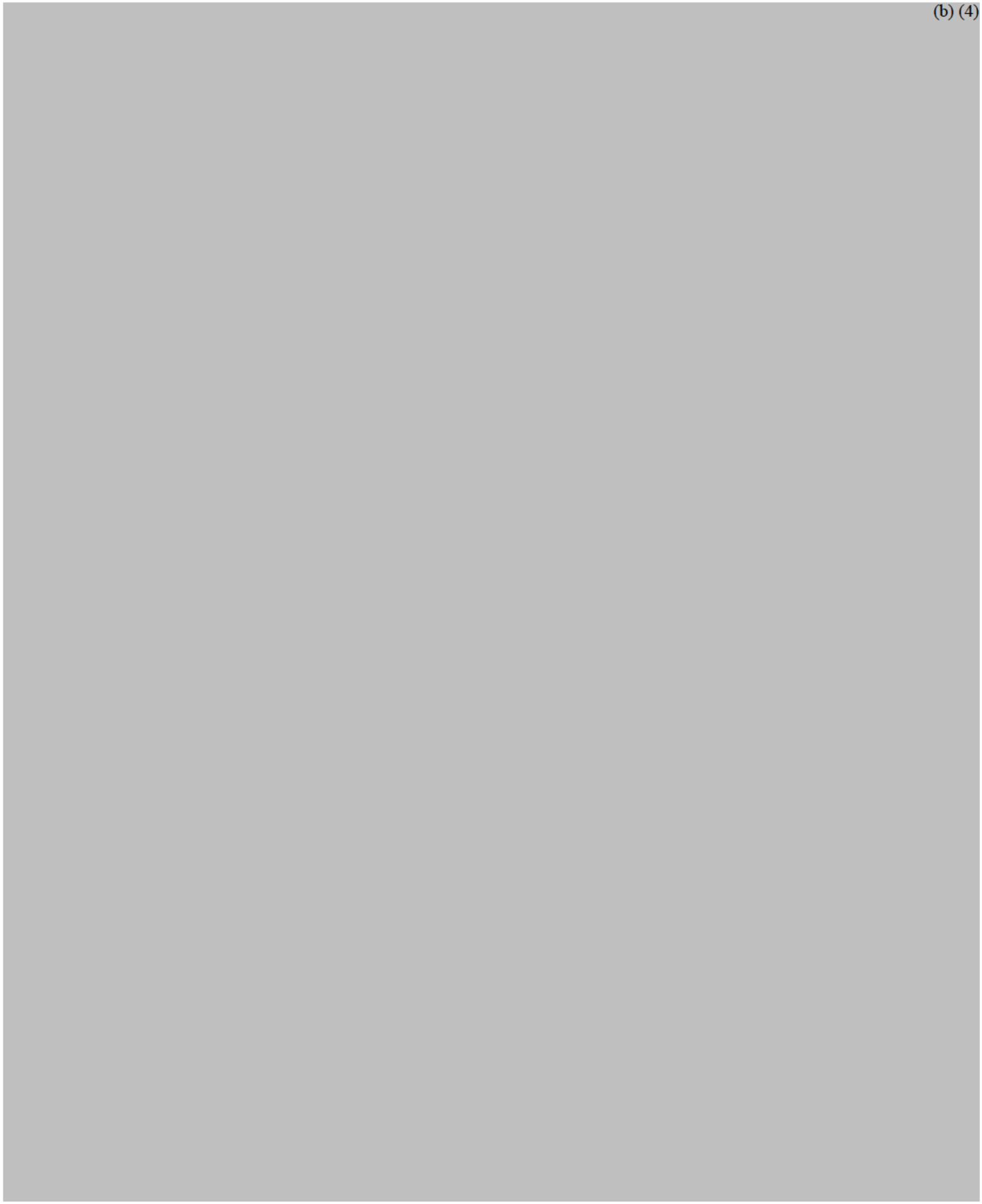

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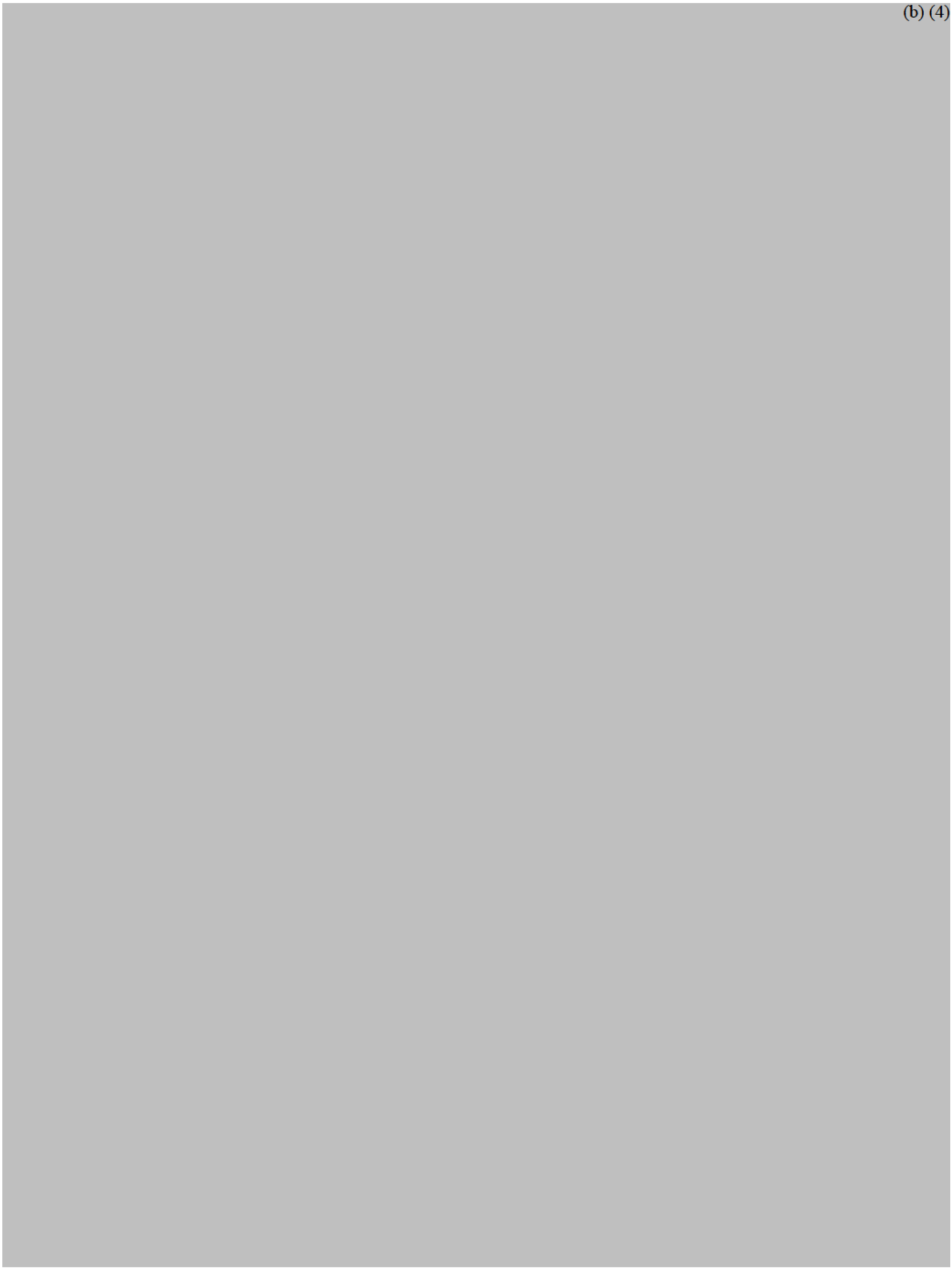

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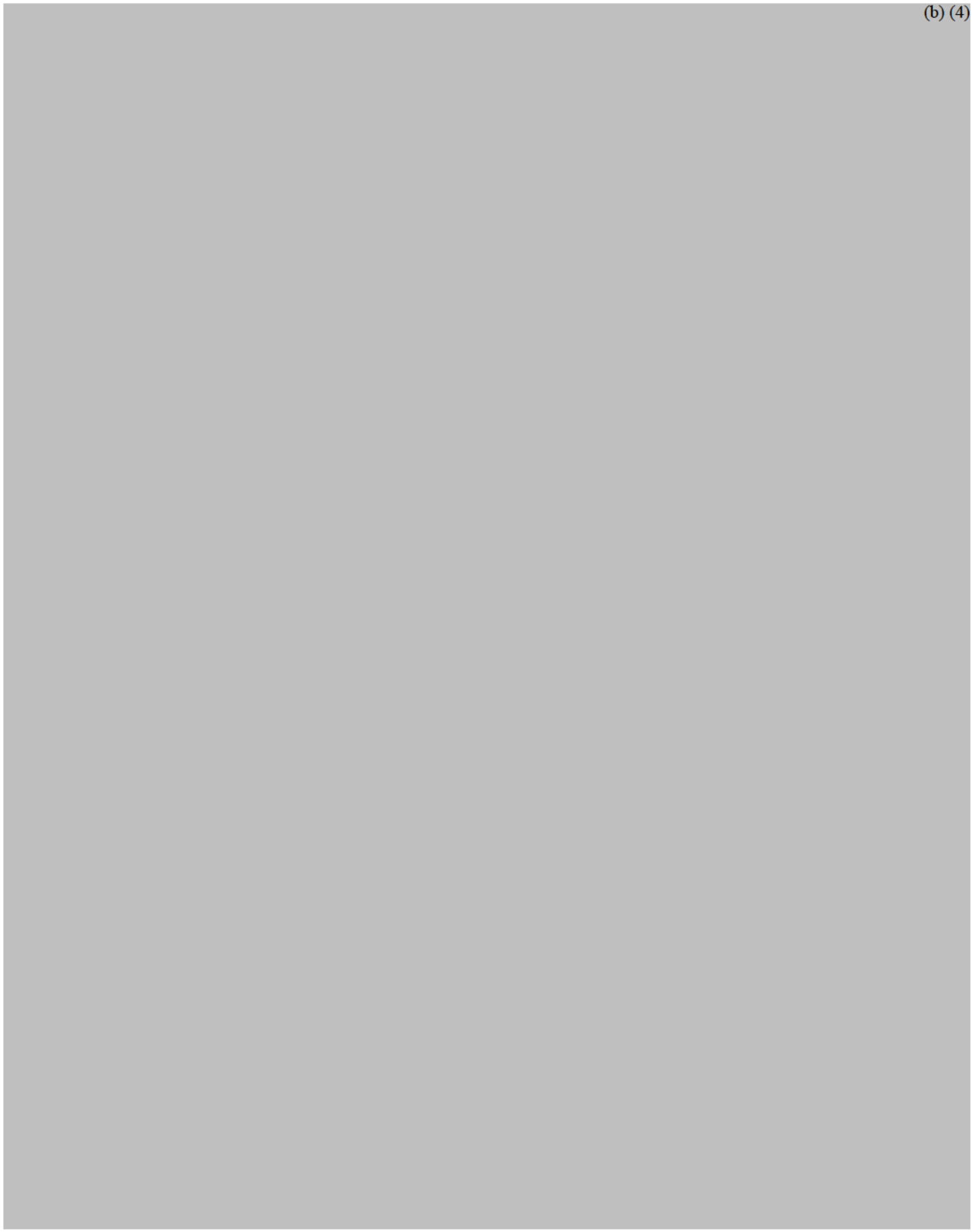
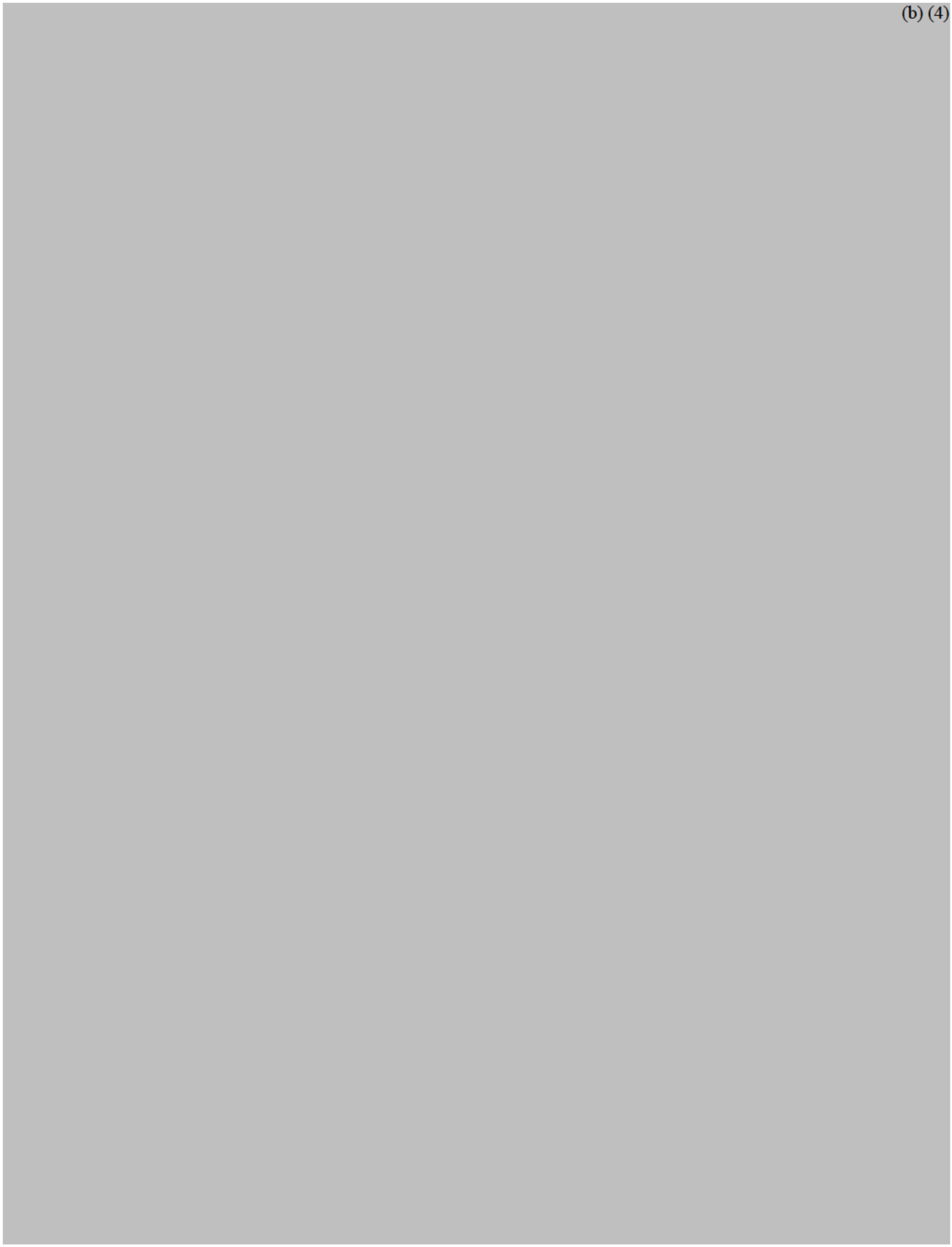


## E-3) Production strain antibiotic susceptibility test

As regards antibiotic resistance, to the knowledge of the CJ, E. coli K12 wild-type strains have not been reported to have any antibiotic resistance. (b) (4) regarding the "antibiotic susceptibility of E.coli (b) (4) E. coli (b) (4) showed same antibiotic sensitivity with E.coli K12 (b) (4) to eight well known antibiotics. These results indicated that there is no possible antibiotic resistance genes in the chromosome of the (b) (4)

(b) (4)



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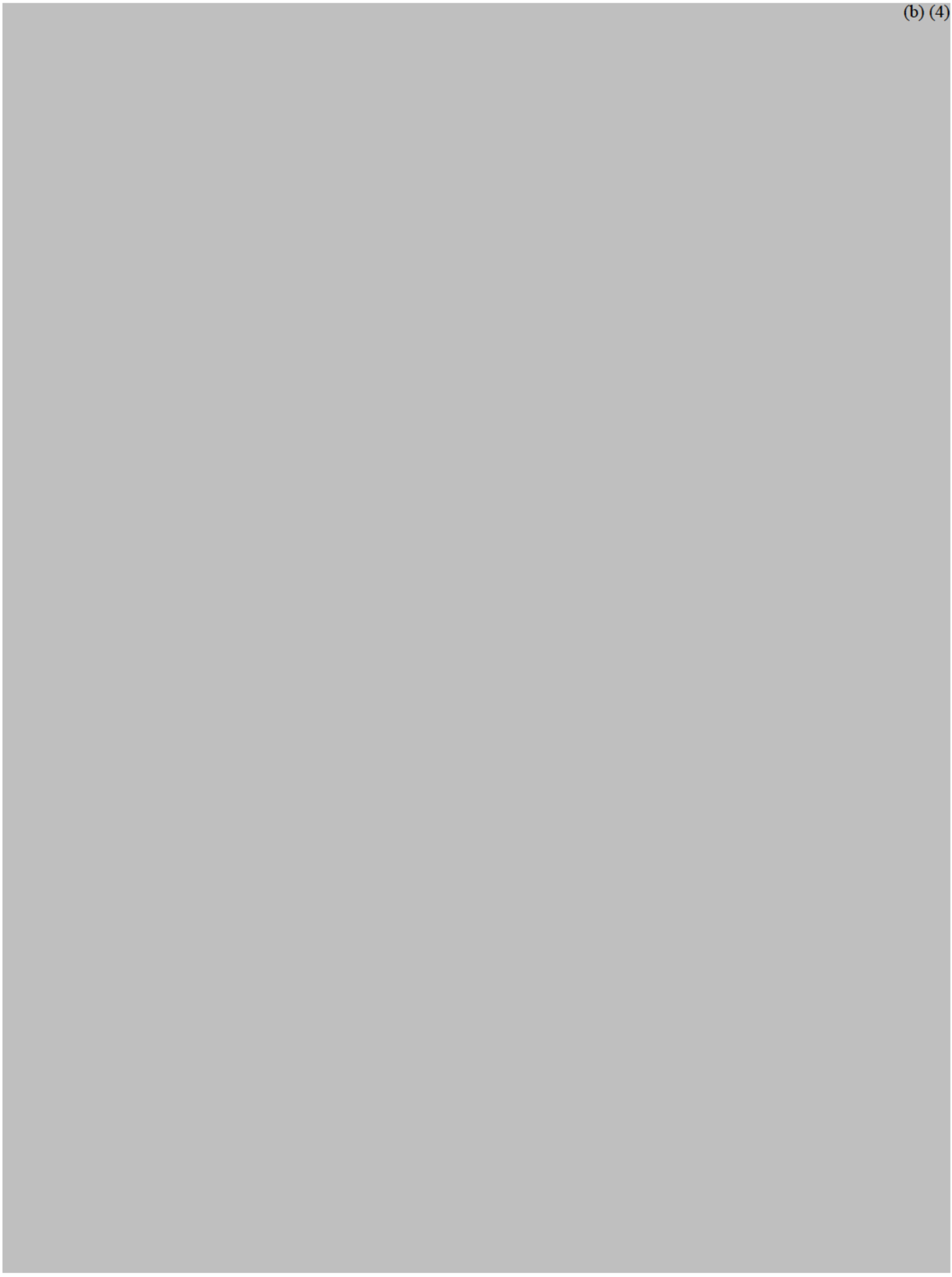

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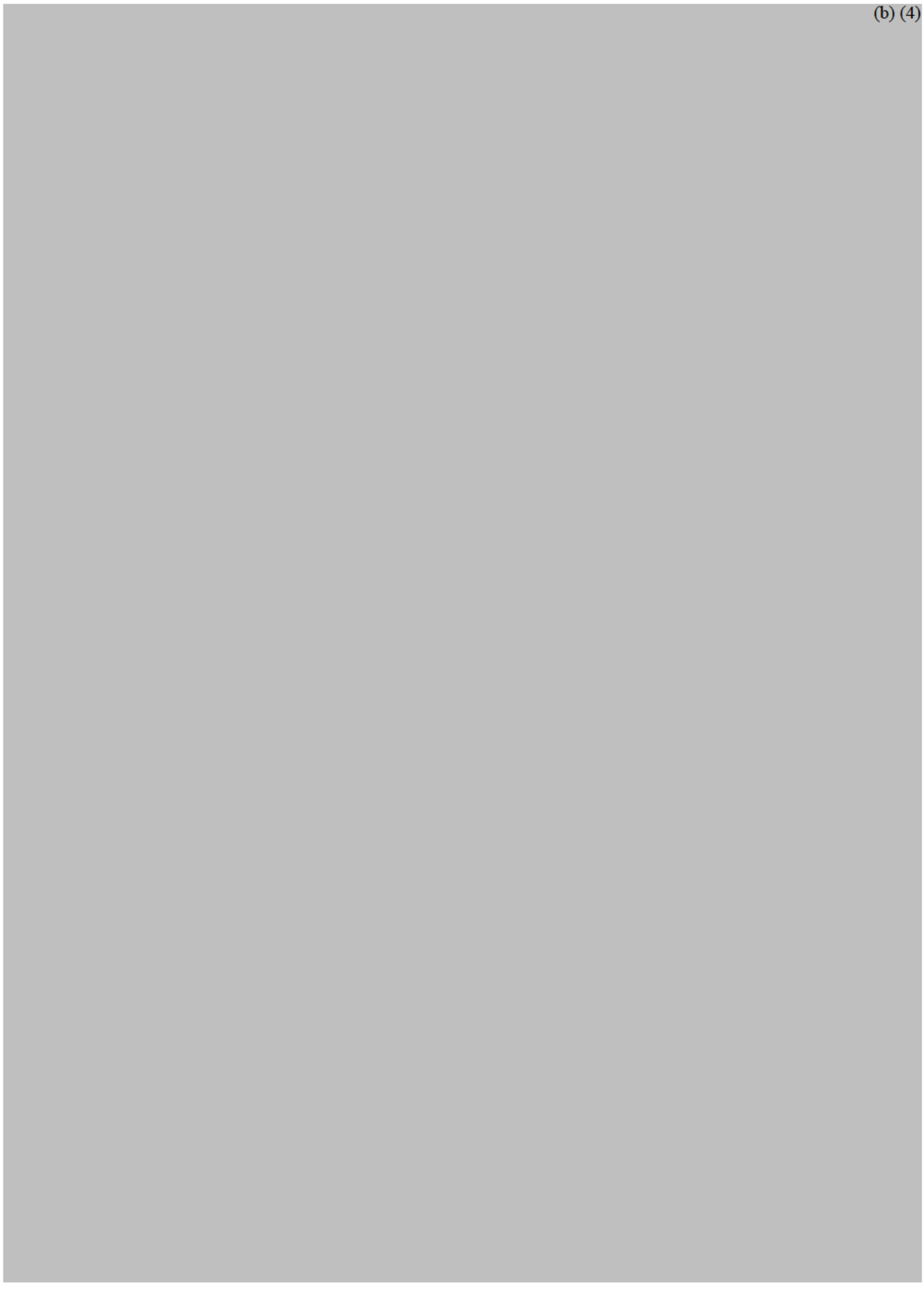

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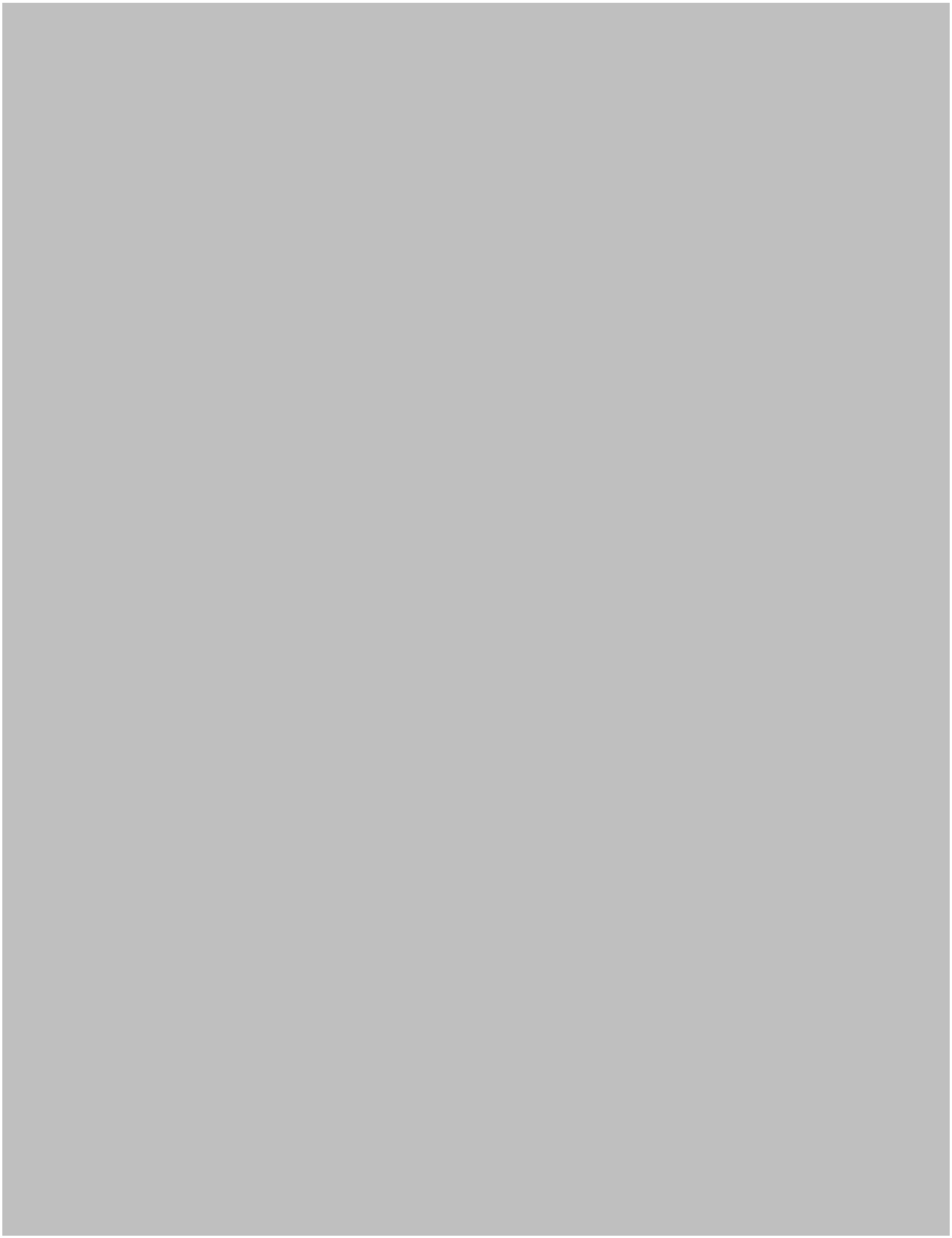

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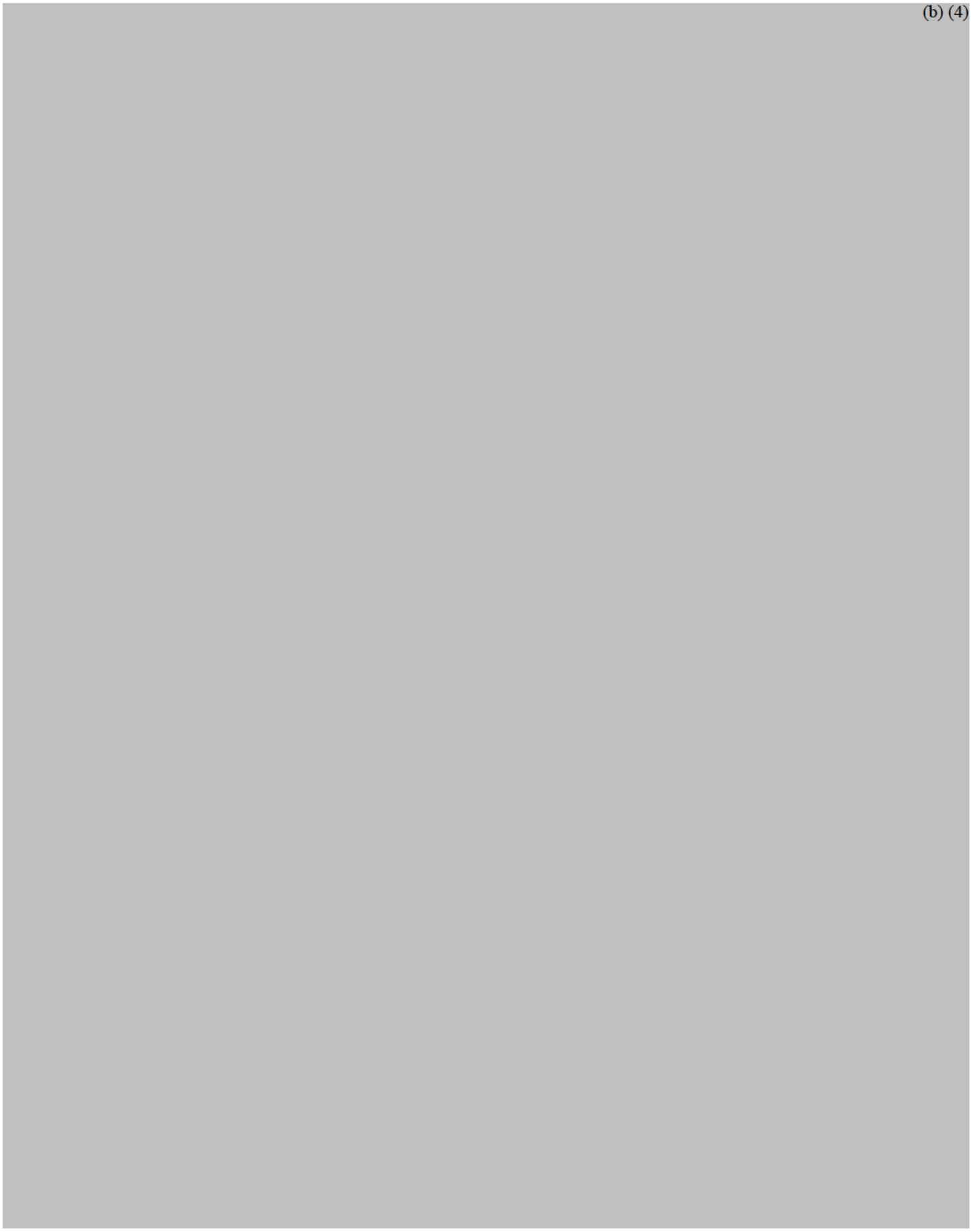

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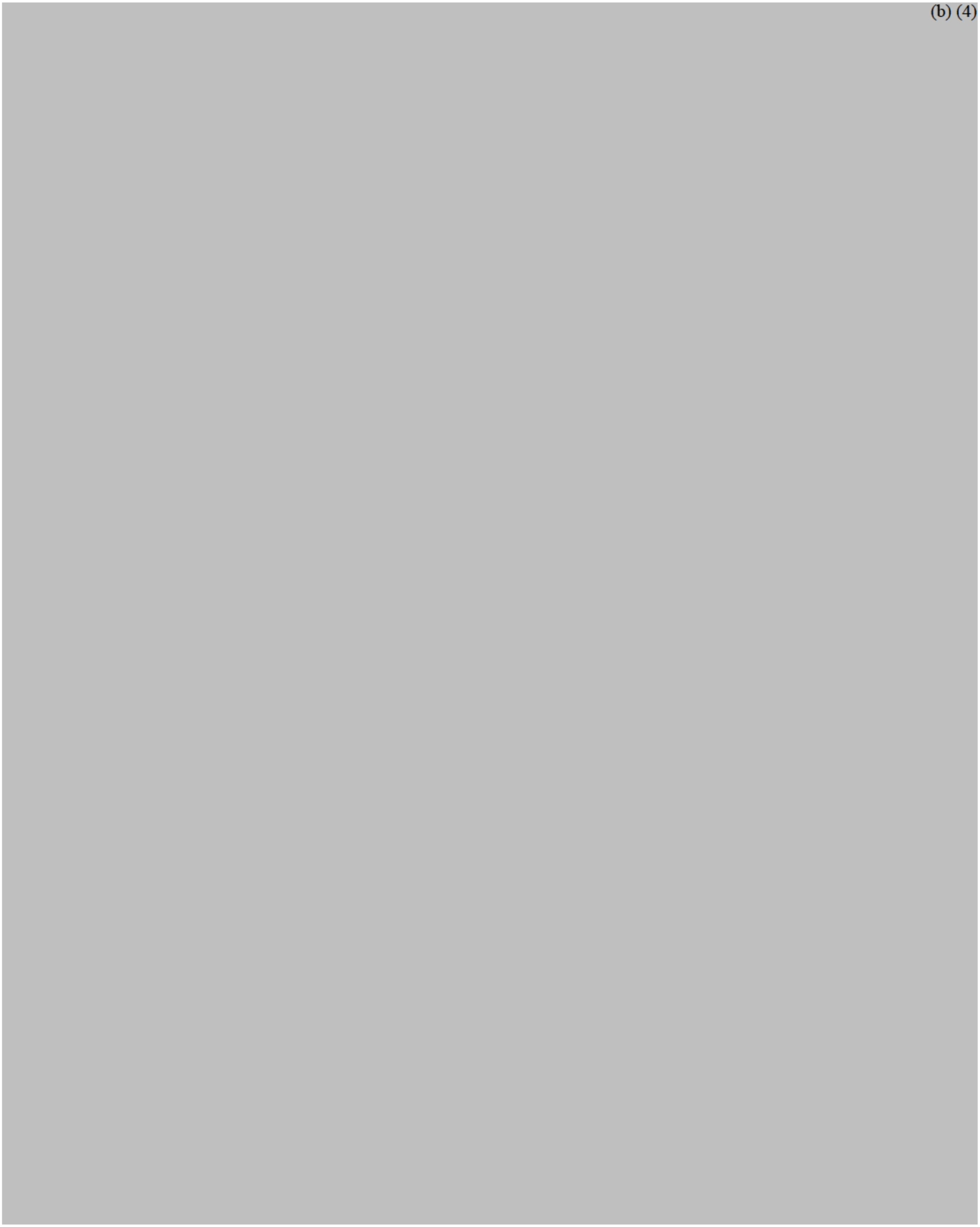
[redacted]

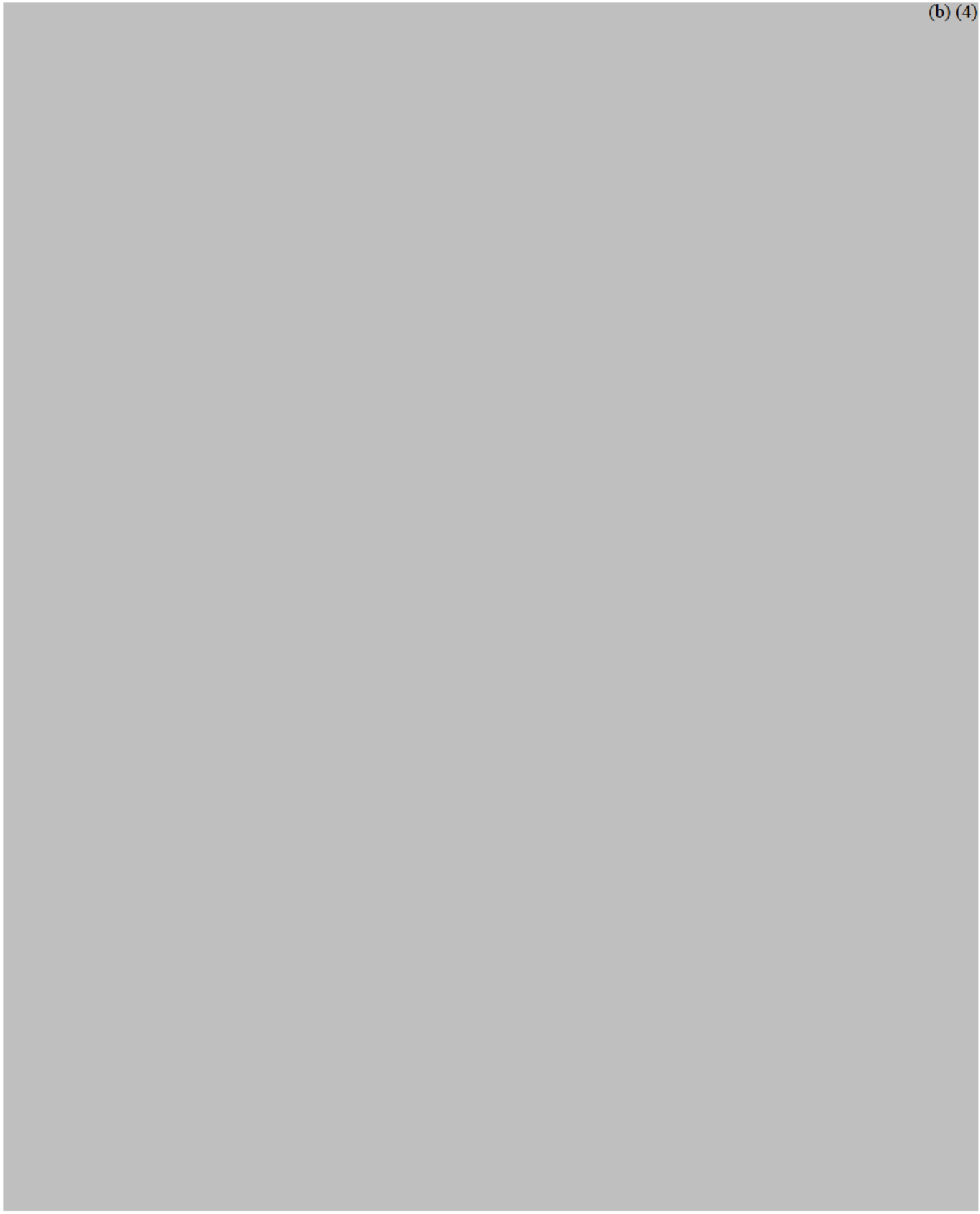
(b) (4)

## E-4) Production strain Mobile element gene test

Most well-known mobile genetic element which has been widely used in genetic recombination in E.coli was selected as a target gene in PCR analysis of mobile genetic element test of Escherichia coli (b) (4). Escherichia coli (b) (4) have been used as the test sample in this experiment. Escherichia coli K12 (b) (4) was used as the negative control and plasmids containing each mobile genetic element were used as the positive control. As the result, the negative control and the genomic DNA of test sample Escherichia coli (b) (4) (b) (4) did not show any band in each PCR reaction. These results indicated that there is no possible mobile genetic element in the chromosome of the test sample. One clear band in each positive sample showed that PCR analysis woks well in each experiment.

(b) (4)

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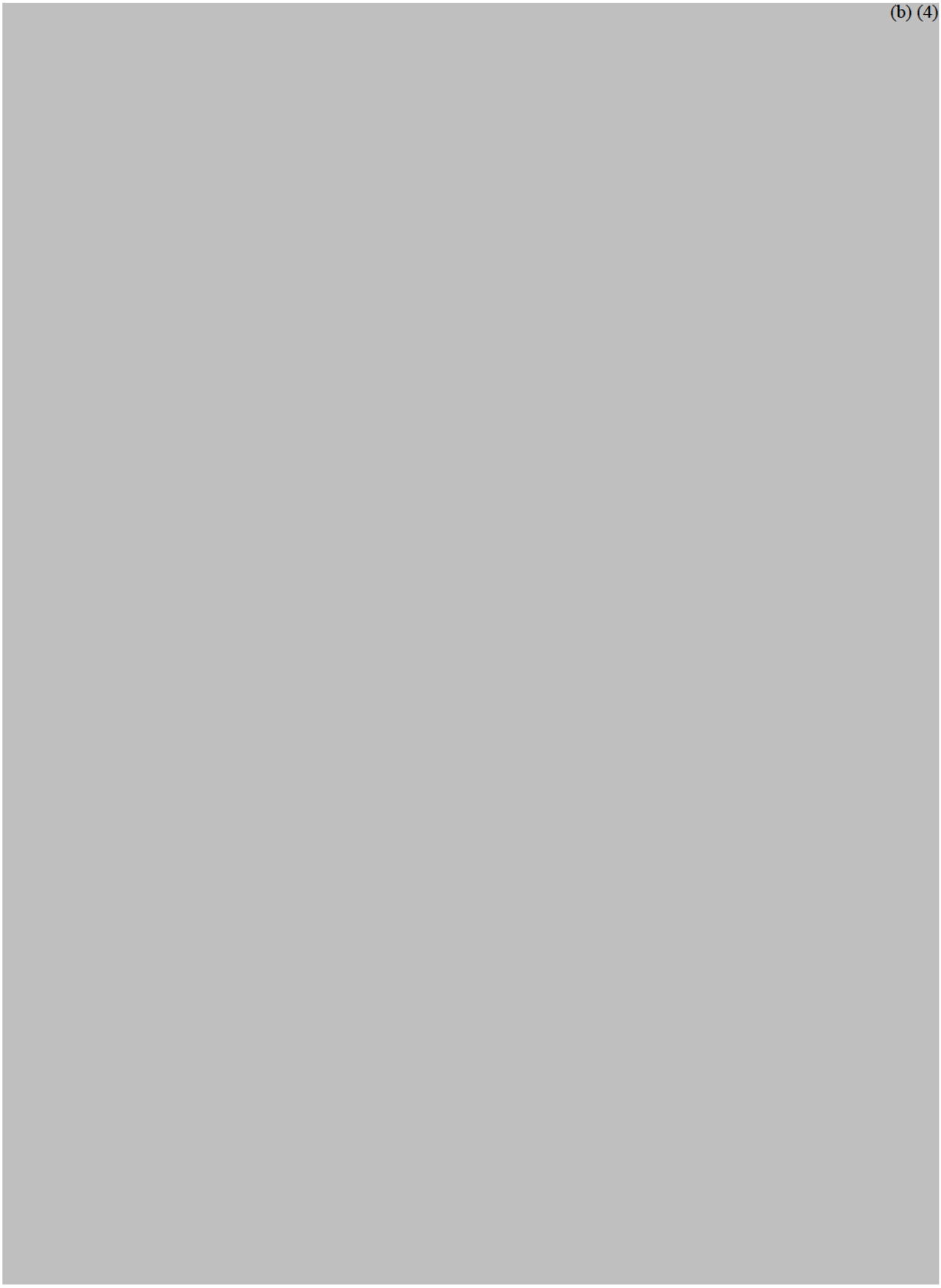


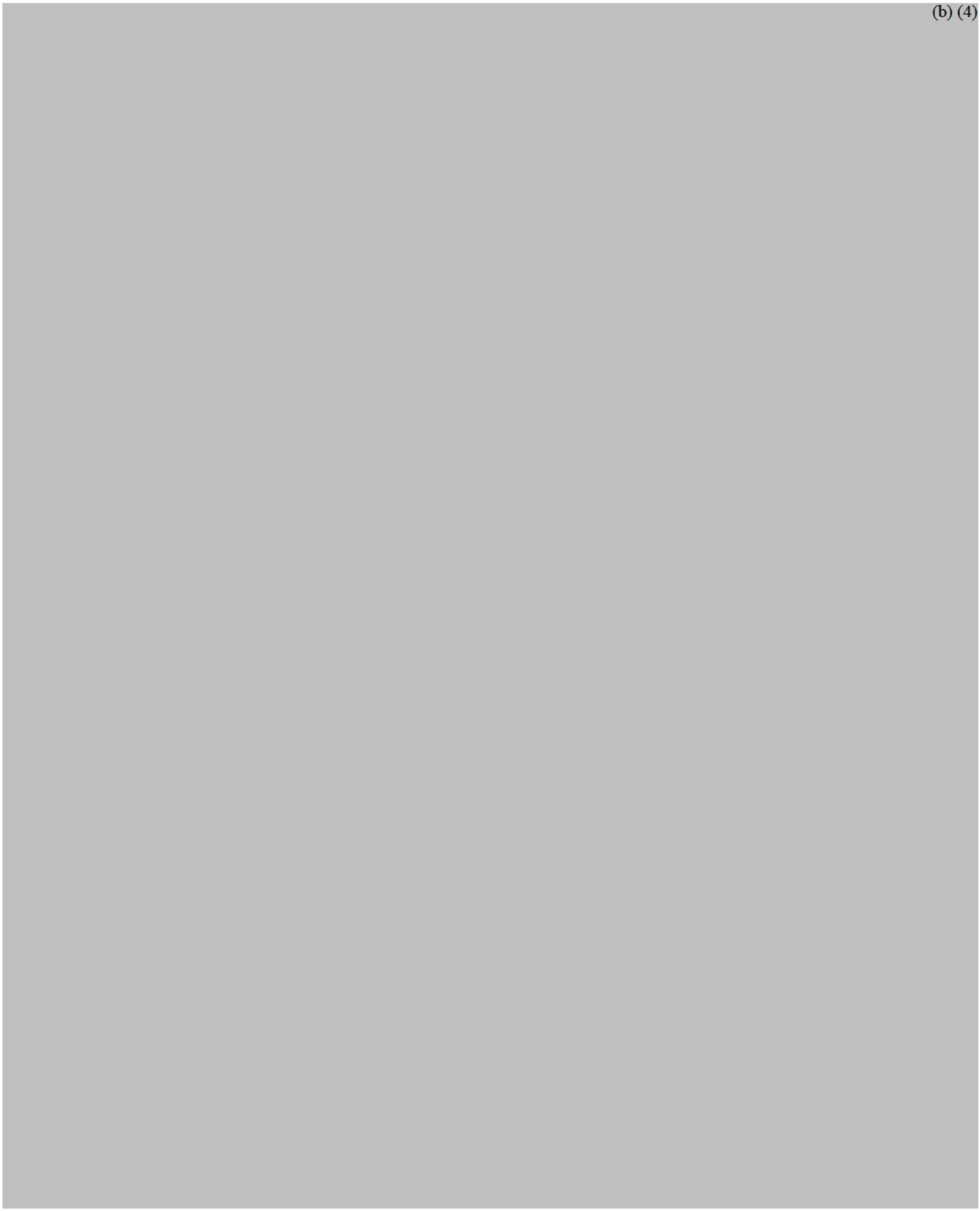
(b) (4)

[redacted]

## E-5) Stability test of production strain


(b) (4) Genomic DNA was prepared from each step of the fermentation, digested by restriction enzymes, and analysed by Southern hybridization analysis using the amplified genes as probe. The amplified genes were confirmed in the same location as the start point of the culture without any genetic location shift.

(b) (4)



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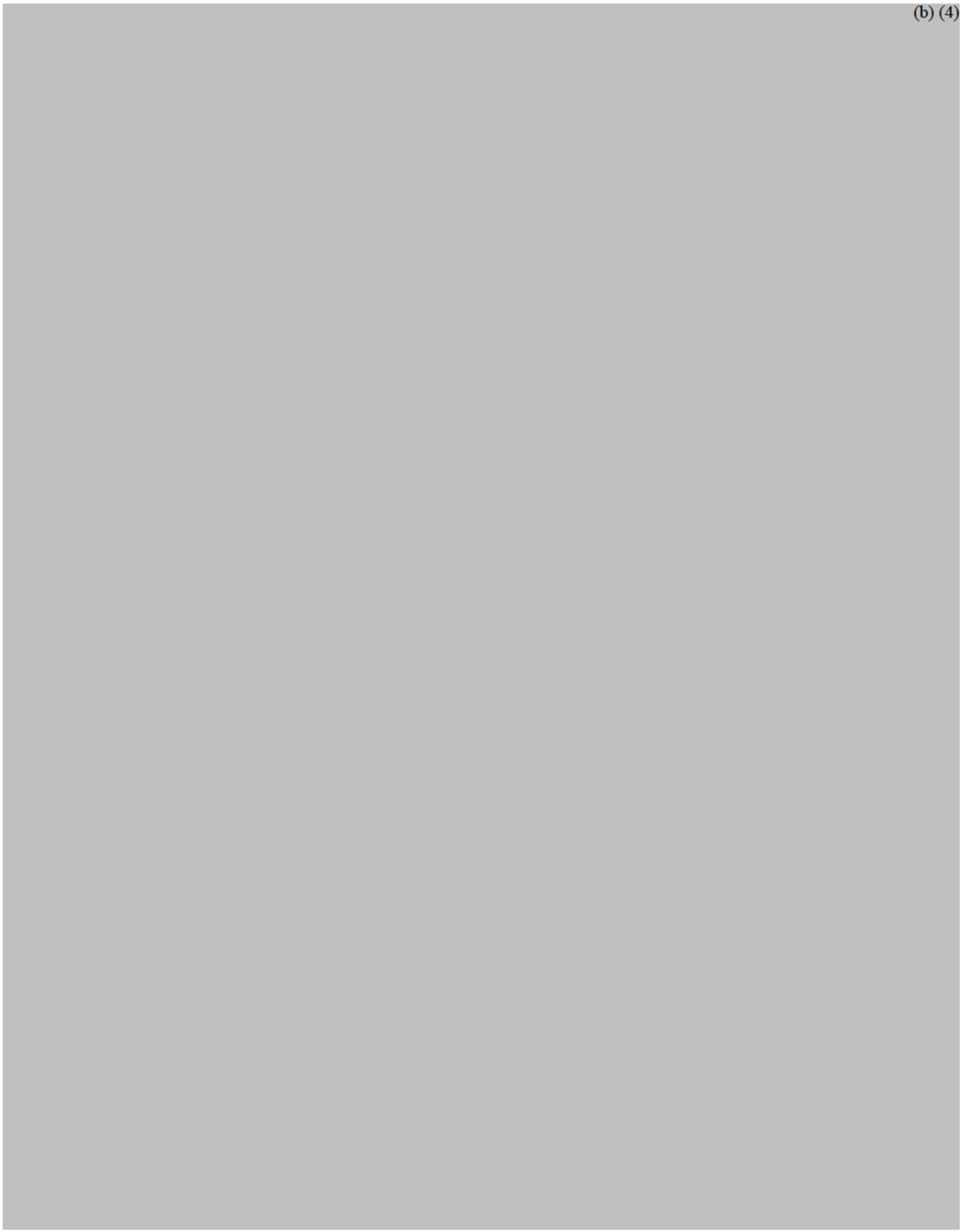

## 1. Summary

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## 2. Test method and Result

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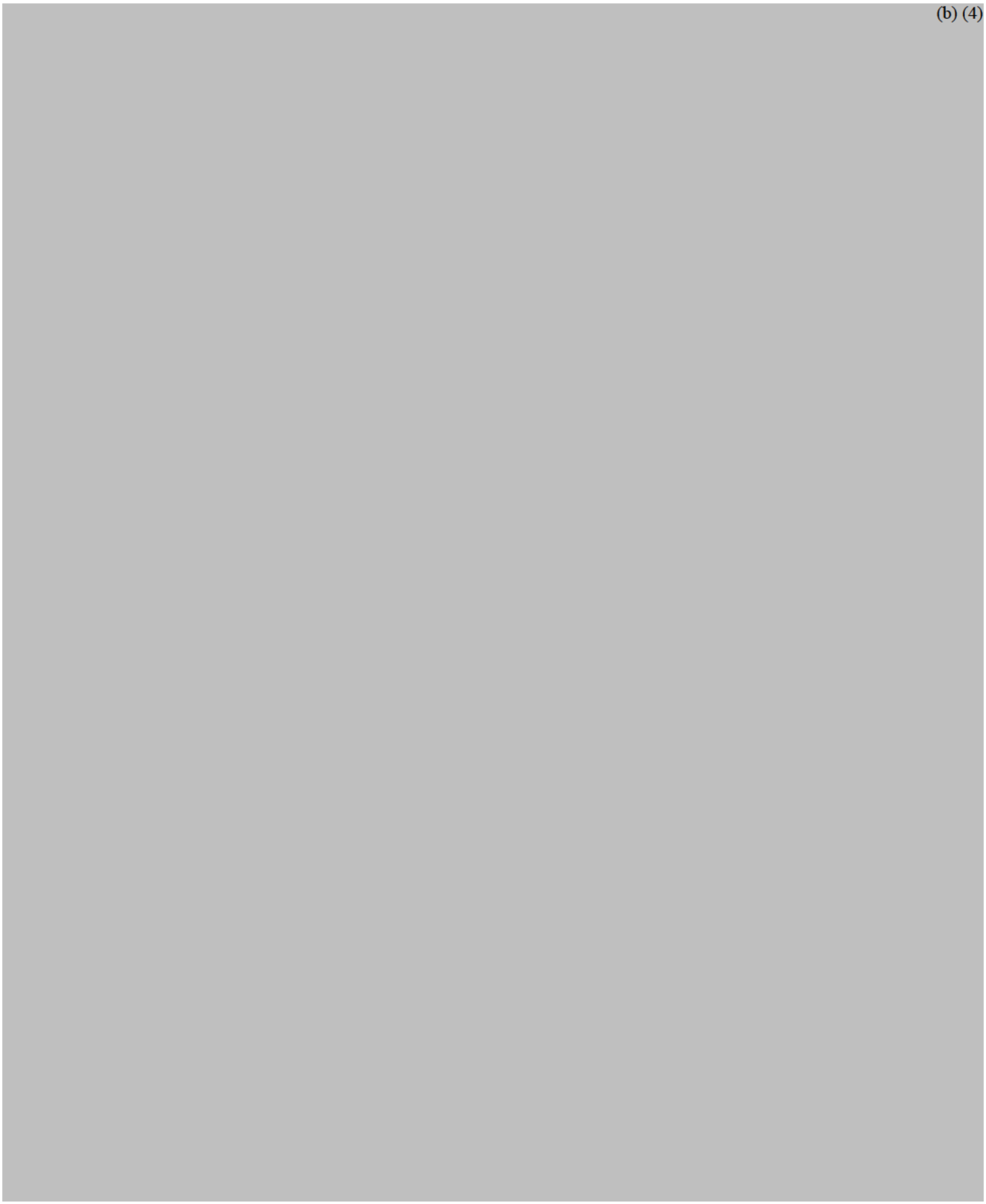
(b) (4)



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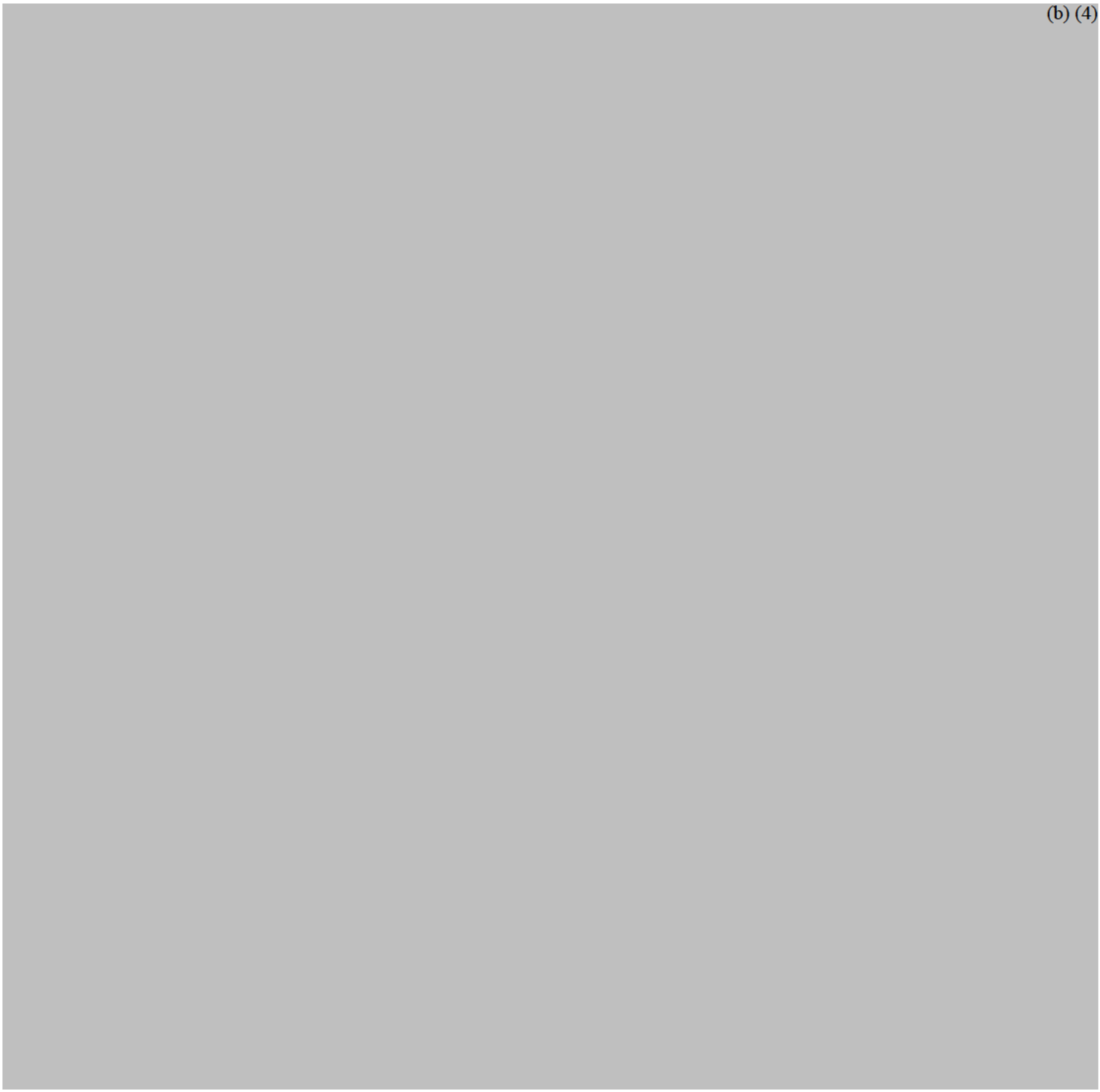

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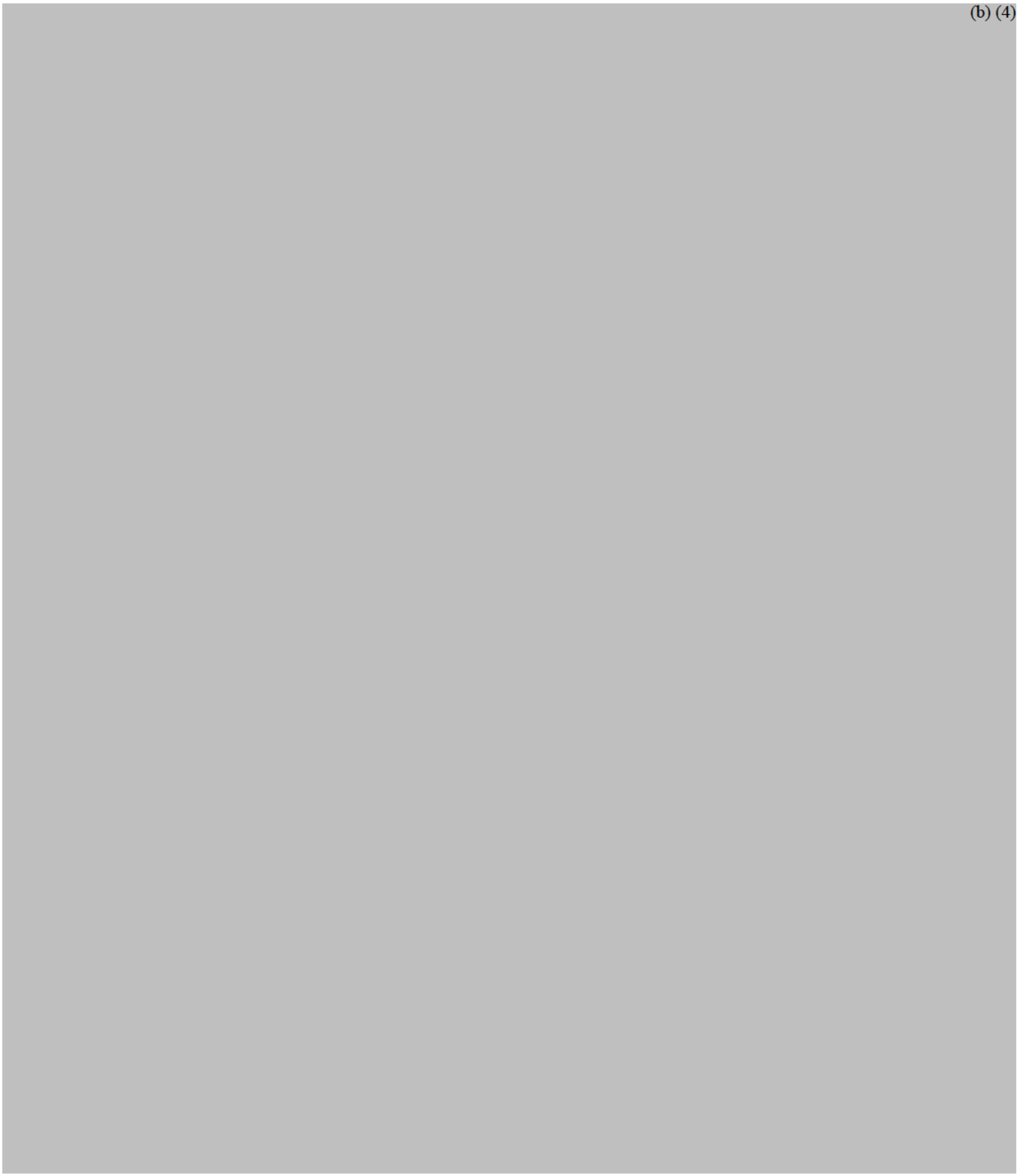

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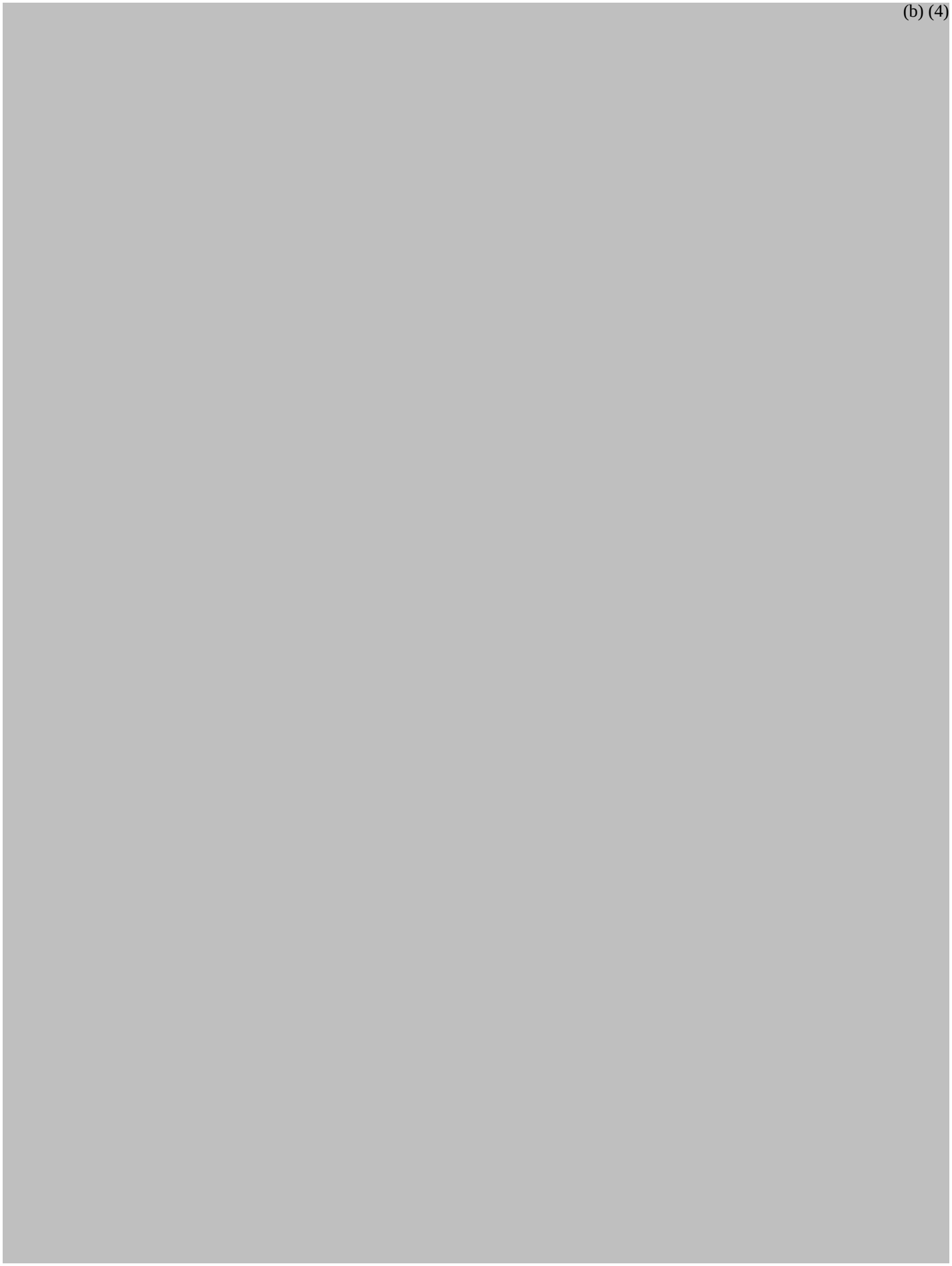

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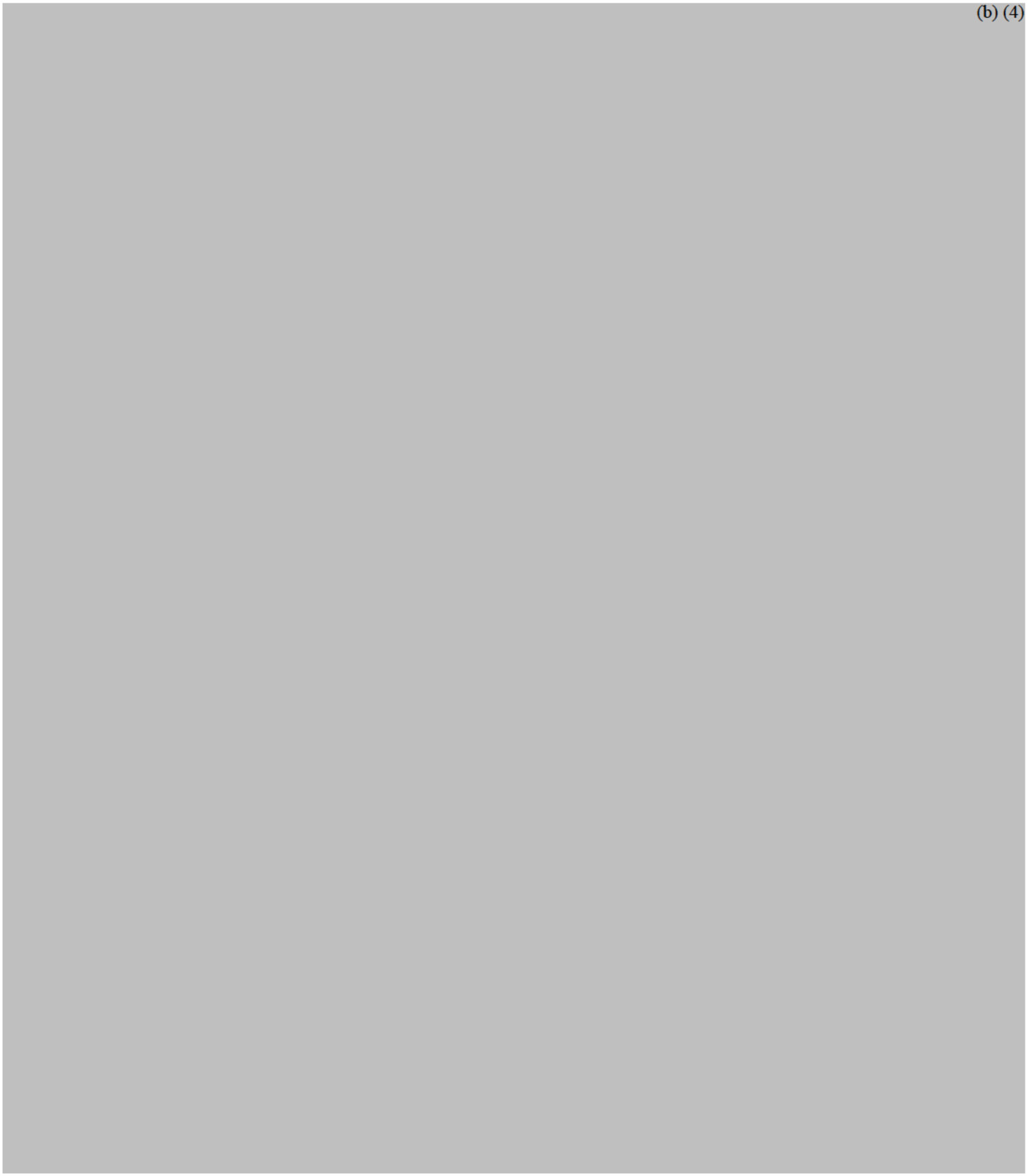


(b) (4)

# REPORT

## The open reading frame analysis

## for the modified site on the (b) (4)

**REPORT DATE: August, 29, 2013**

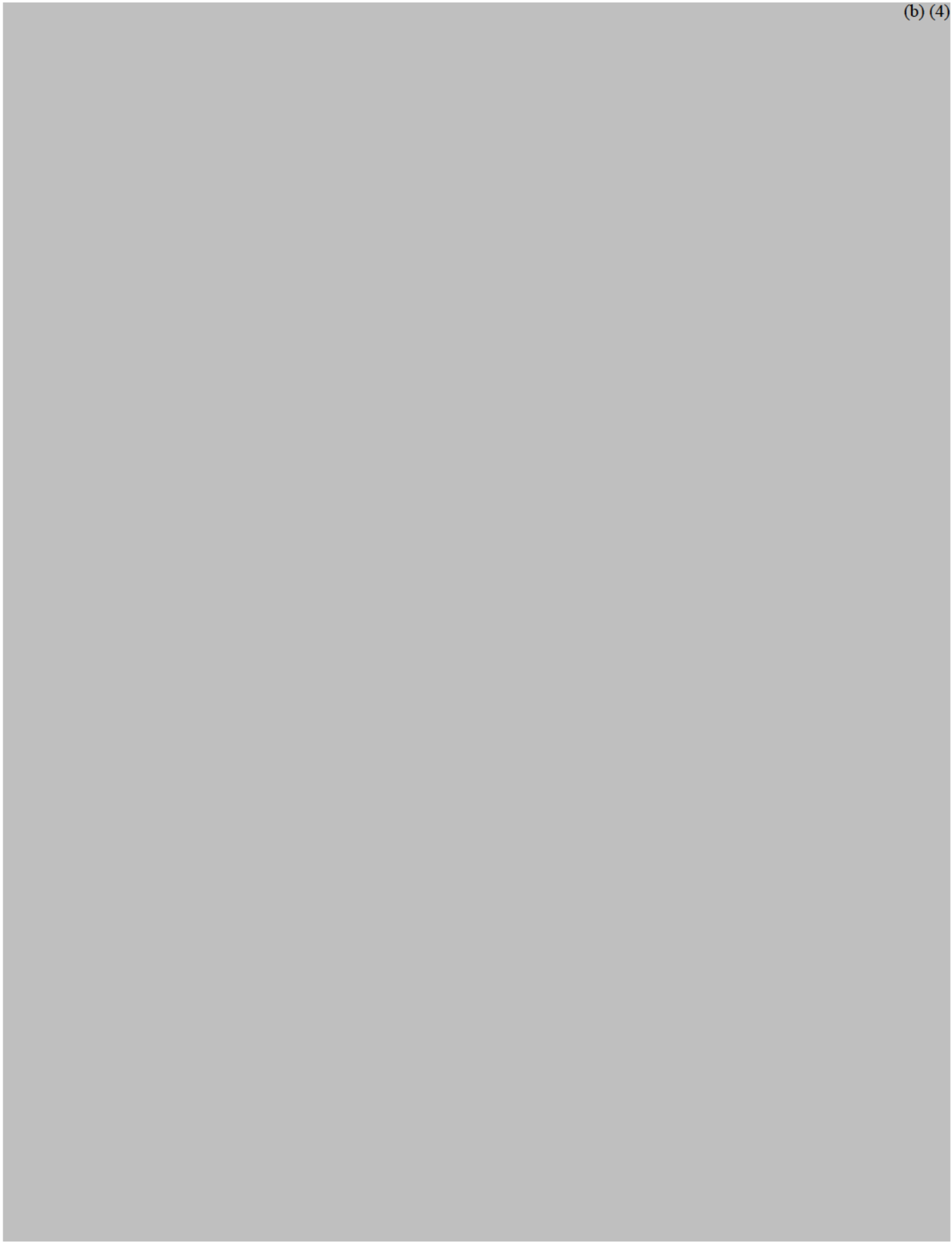
**CJ CheilJedang Research Institute of Biotechnology**

## 1. Summary

(b) (4)

(b) (4) It proved that there is no protein expression which was not intended.

(b) (4)



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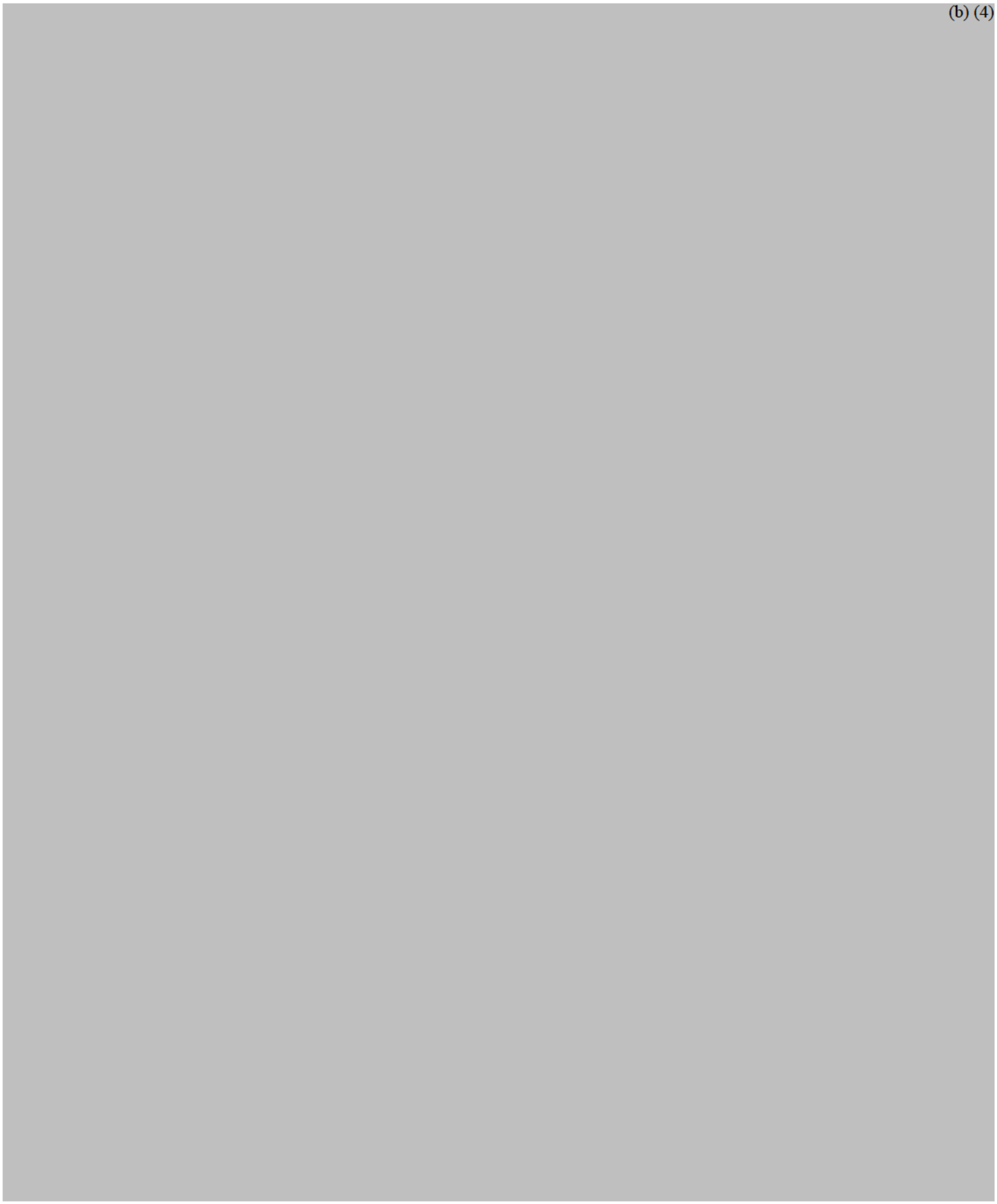

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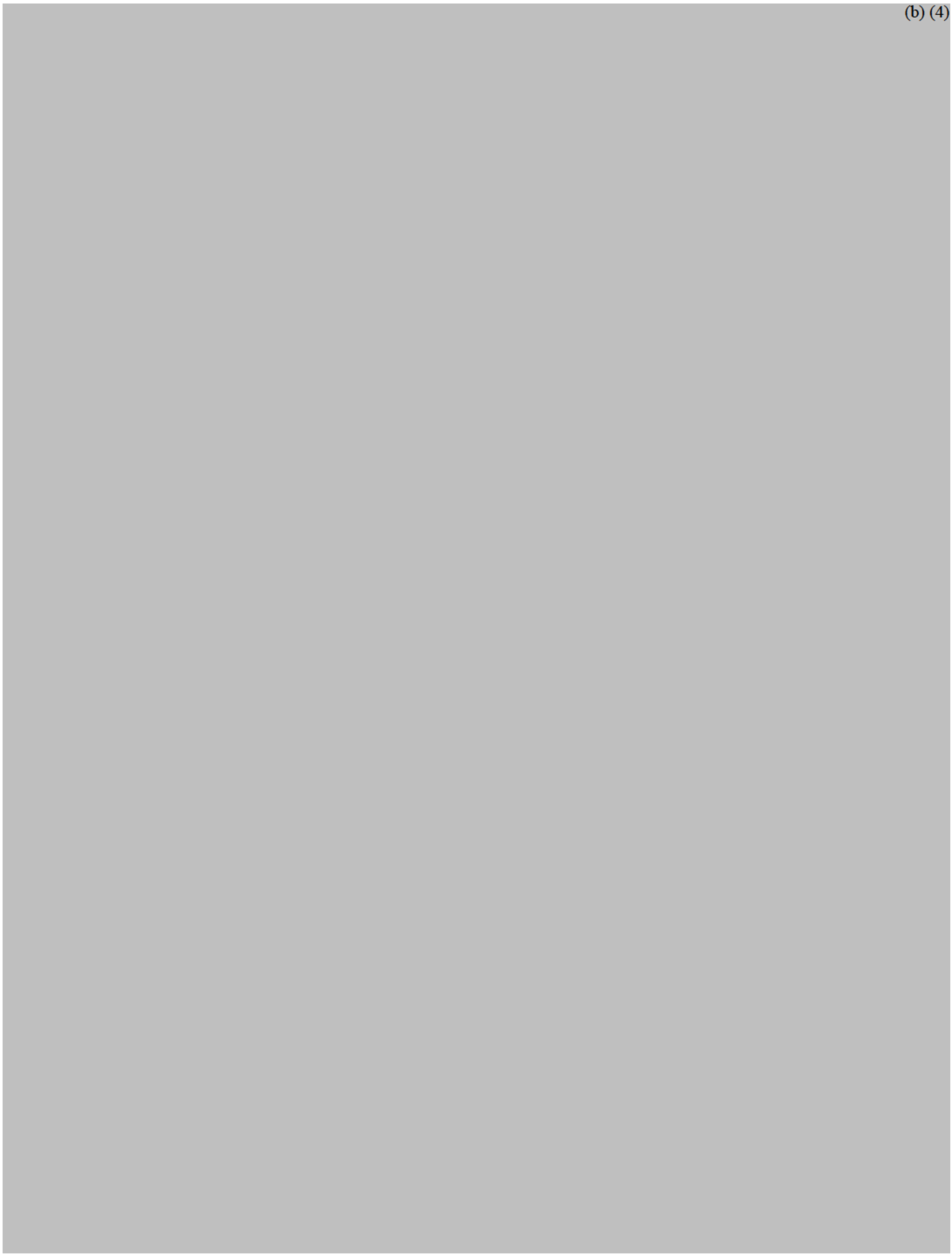

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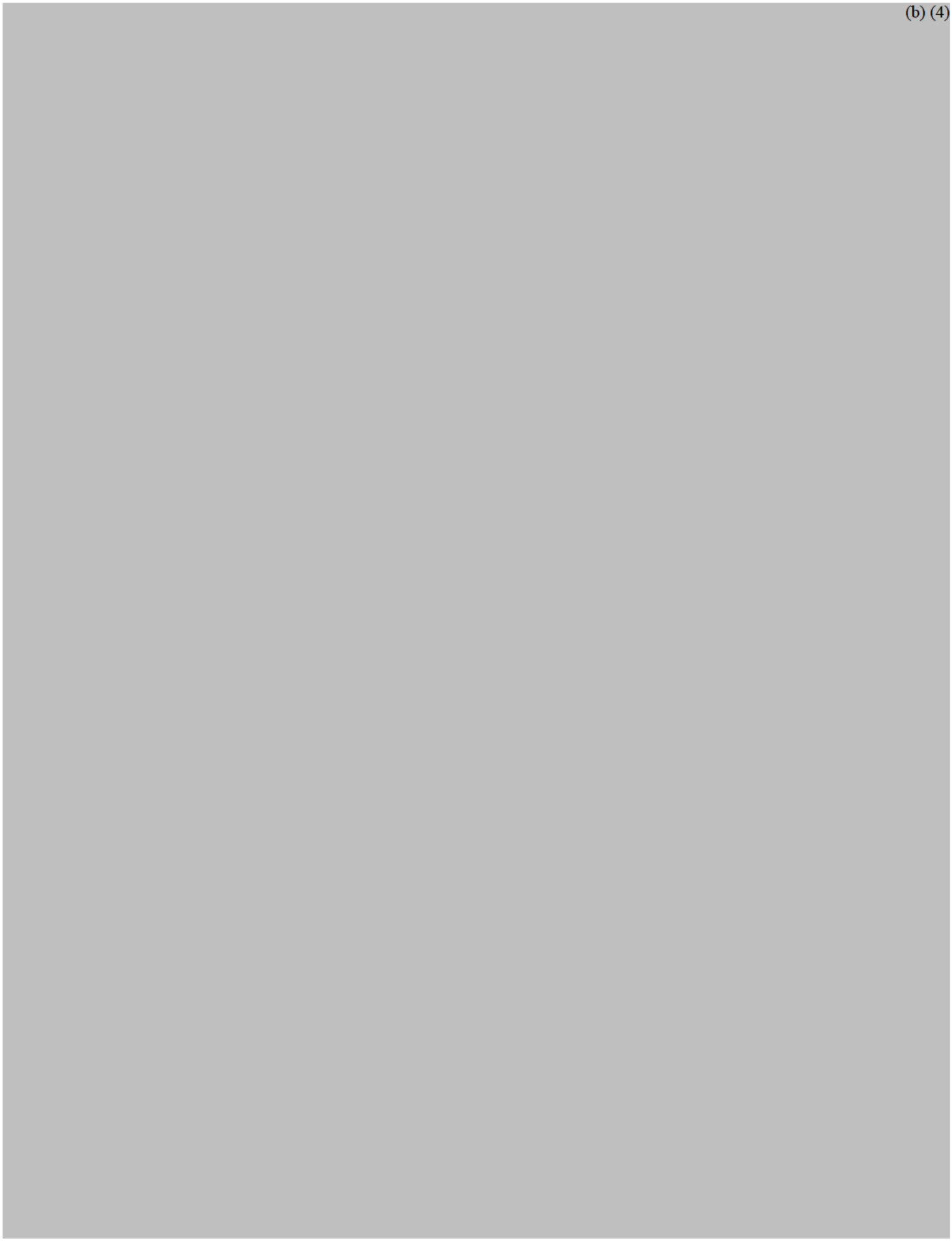

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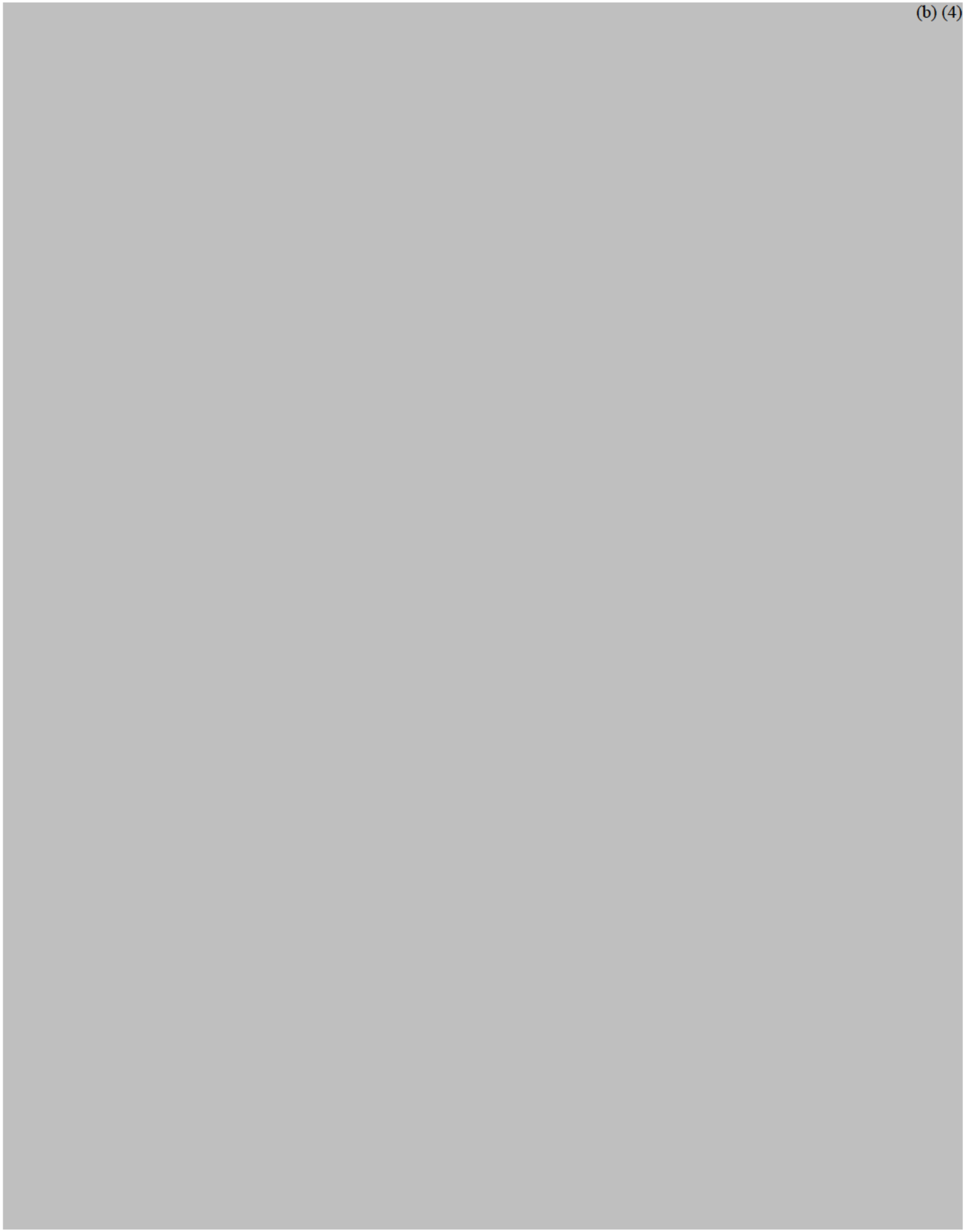

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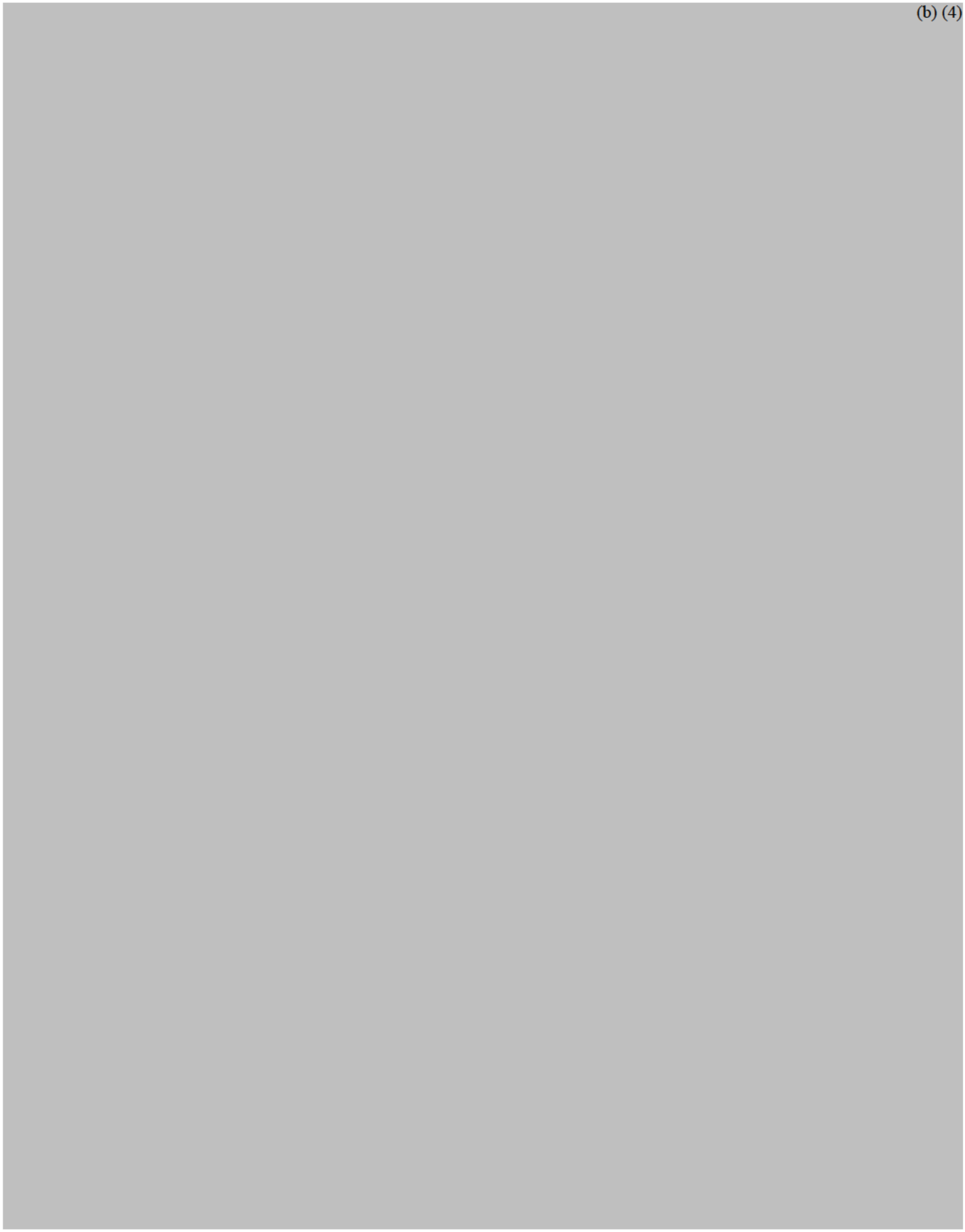

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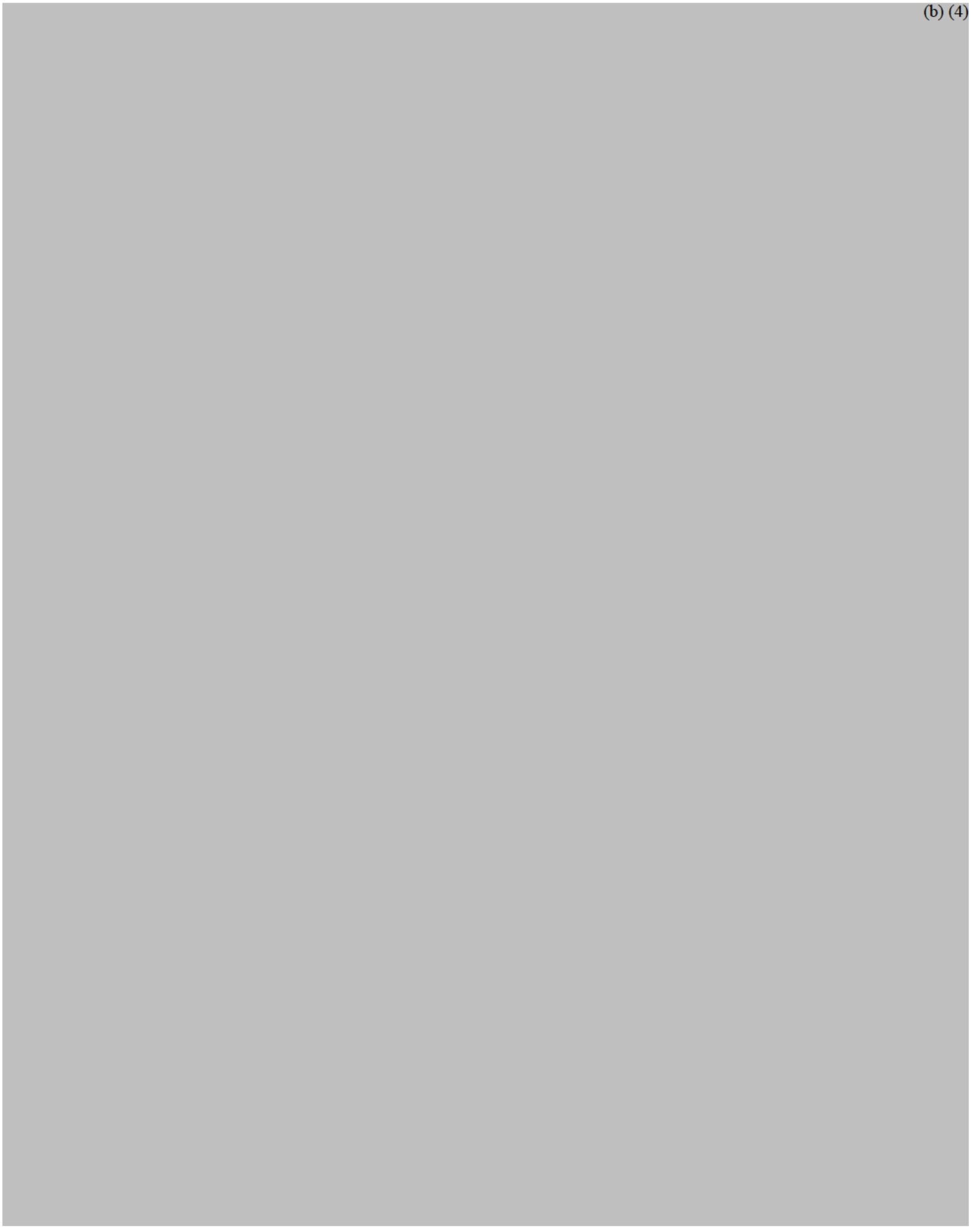

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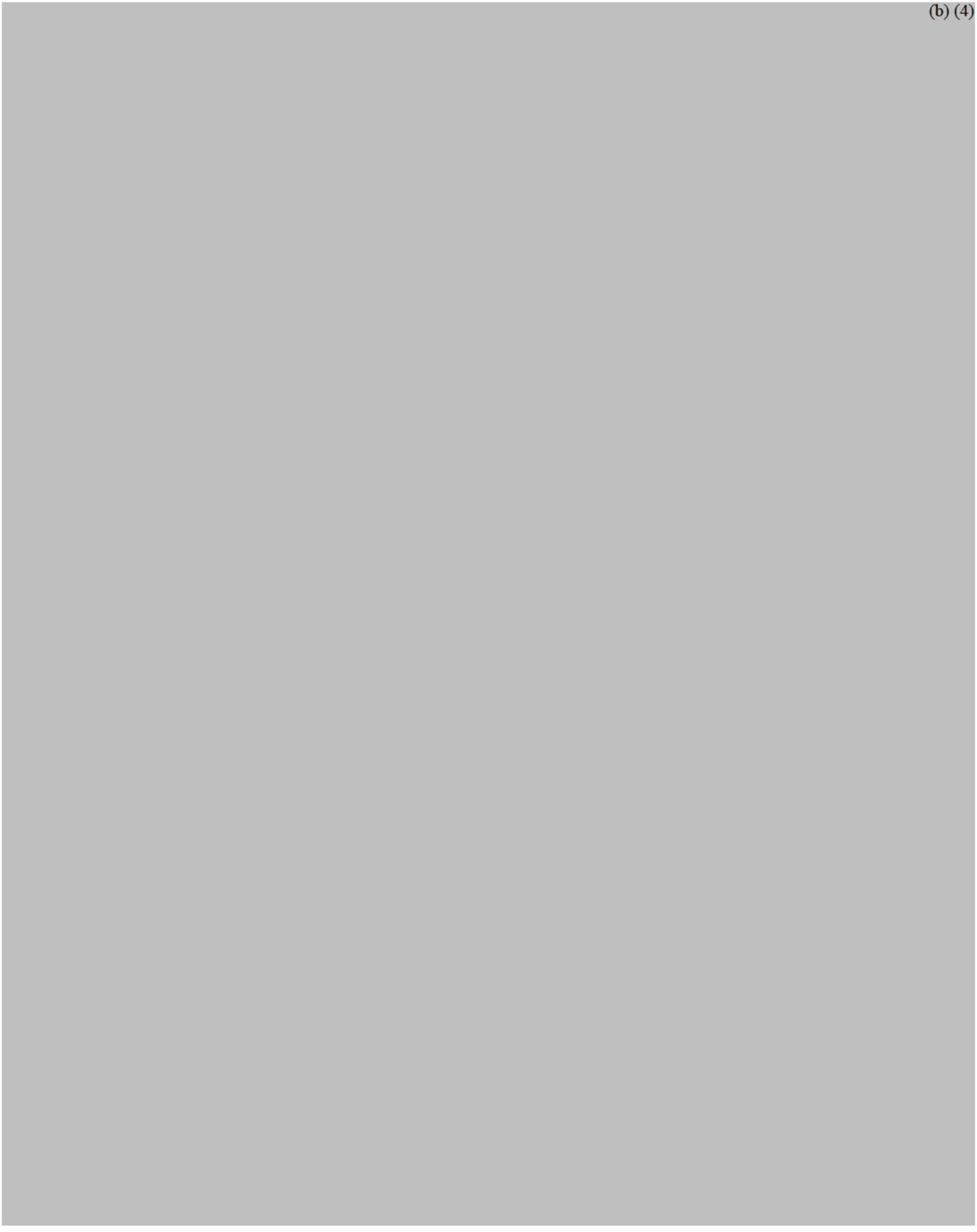

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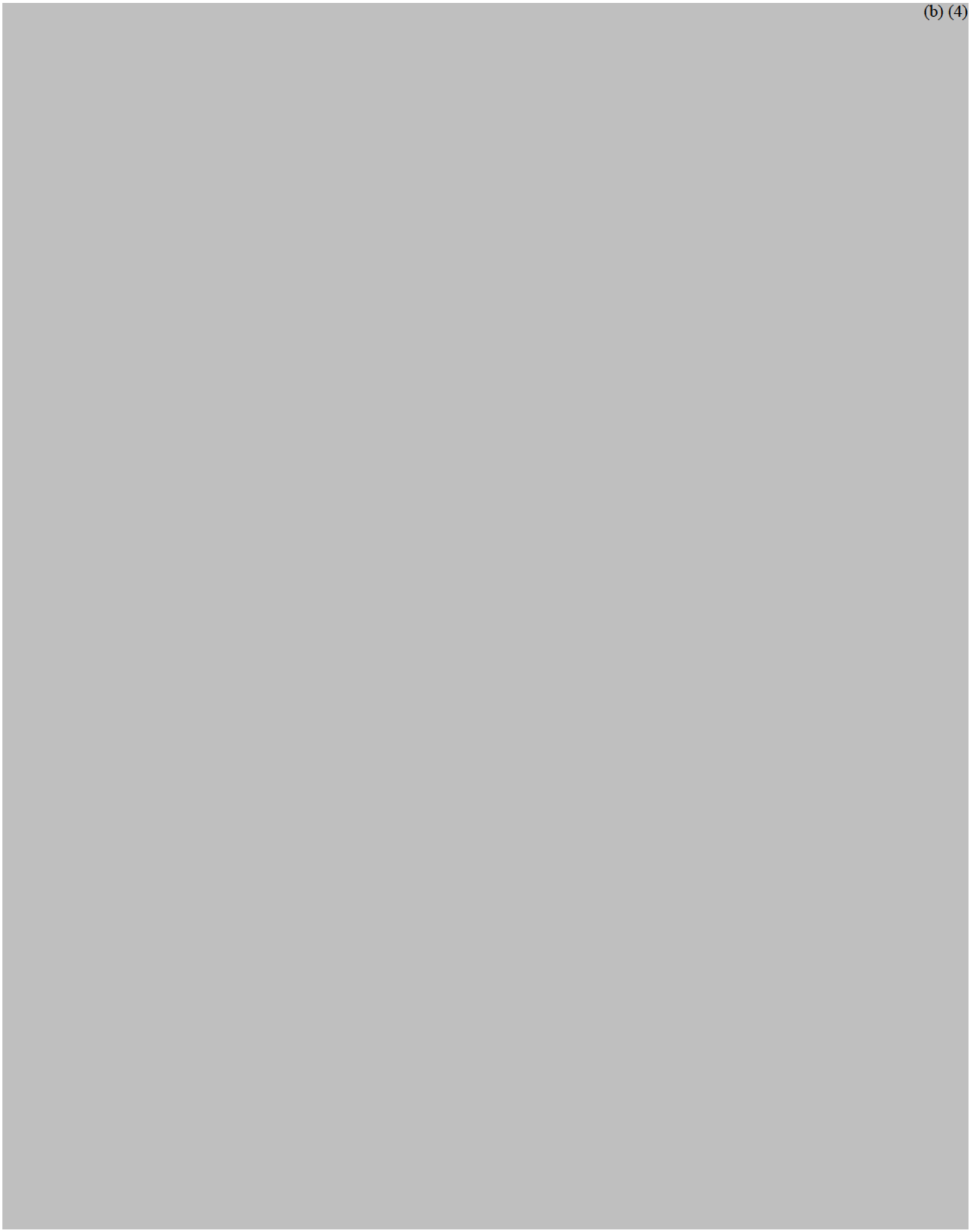

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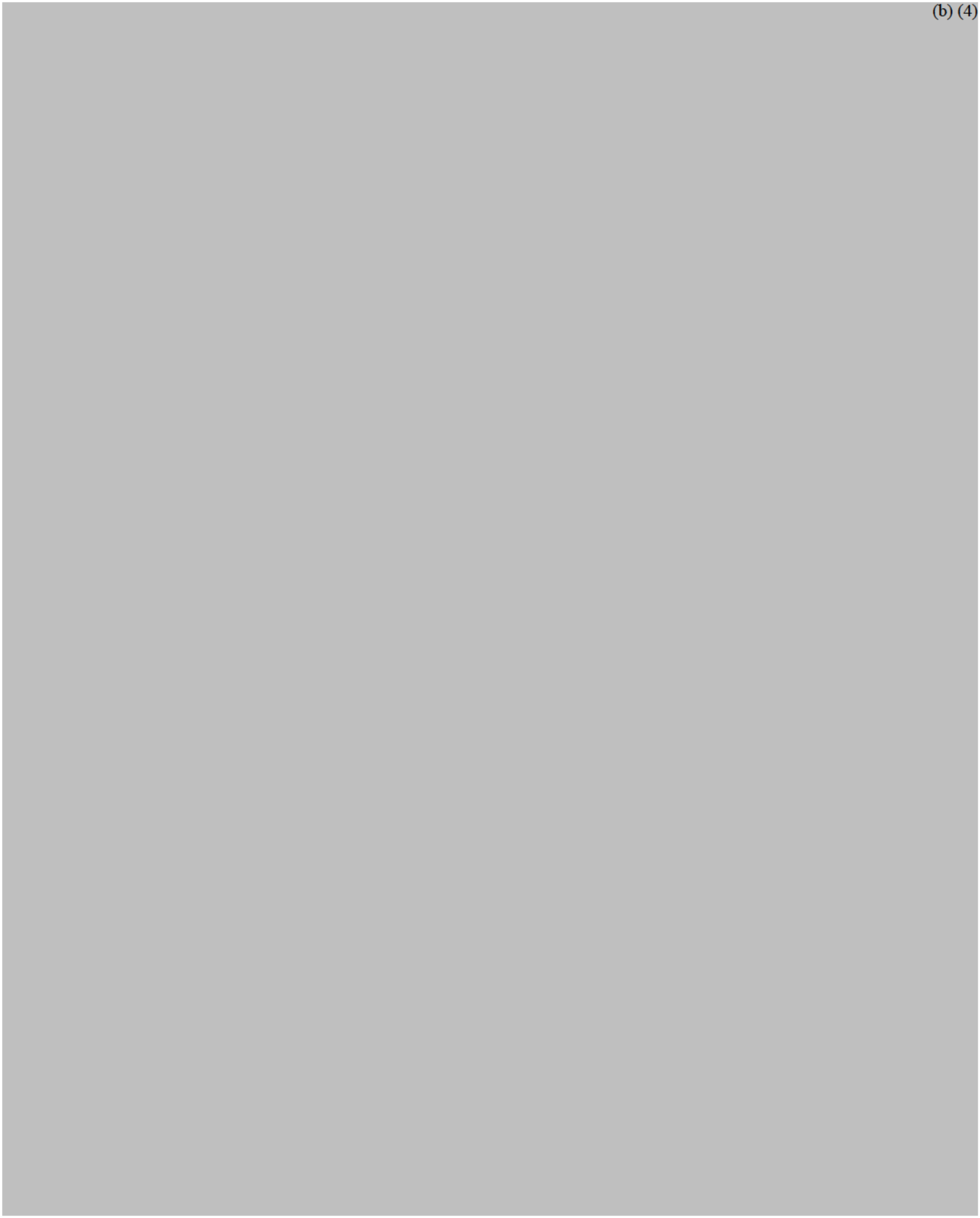

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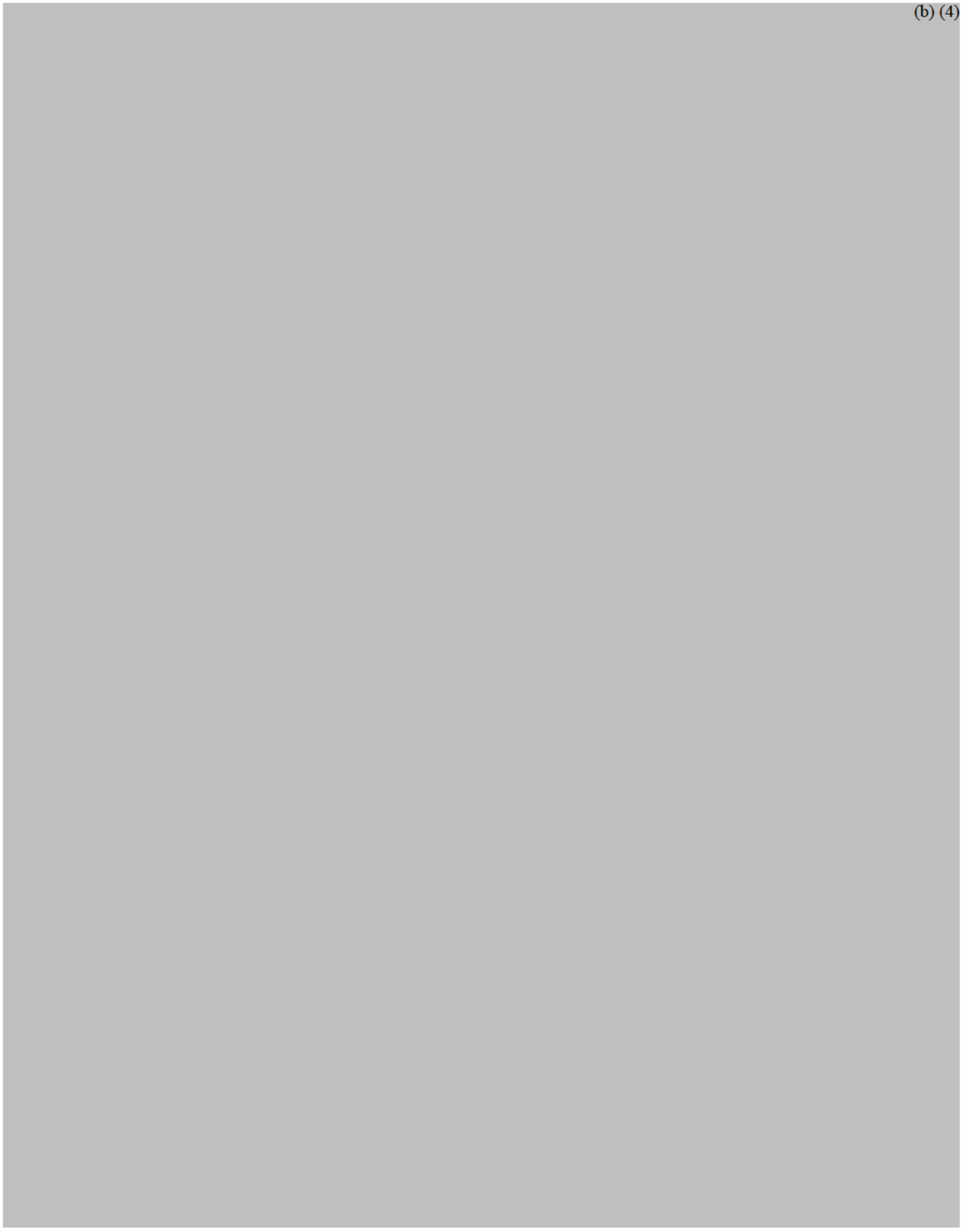

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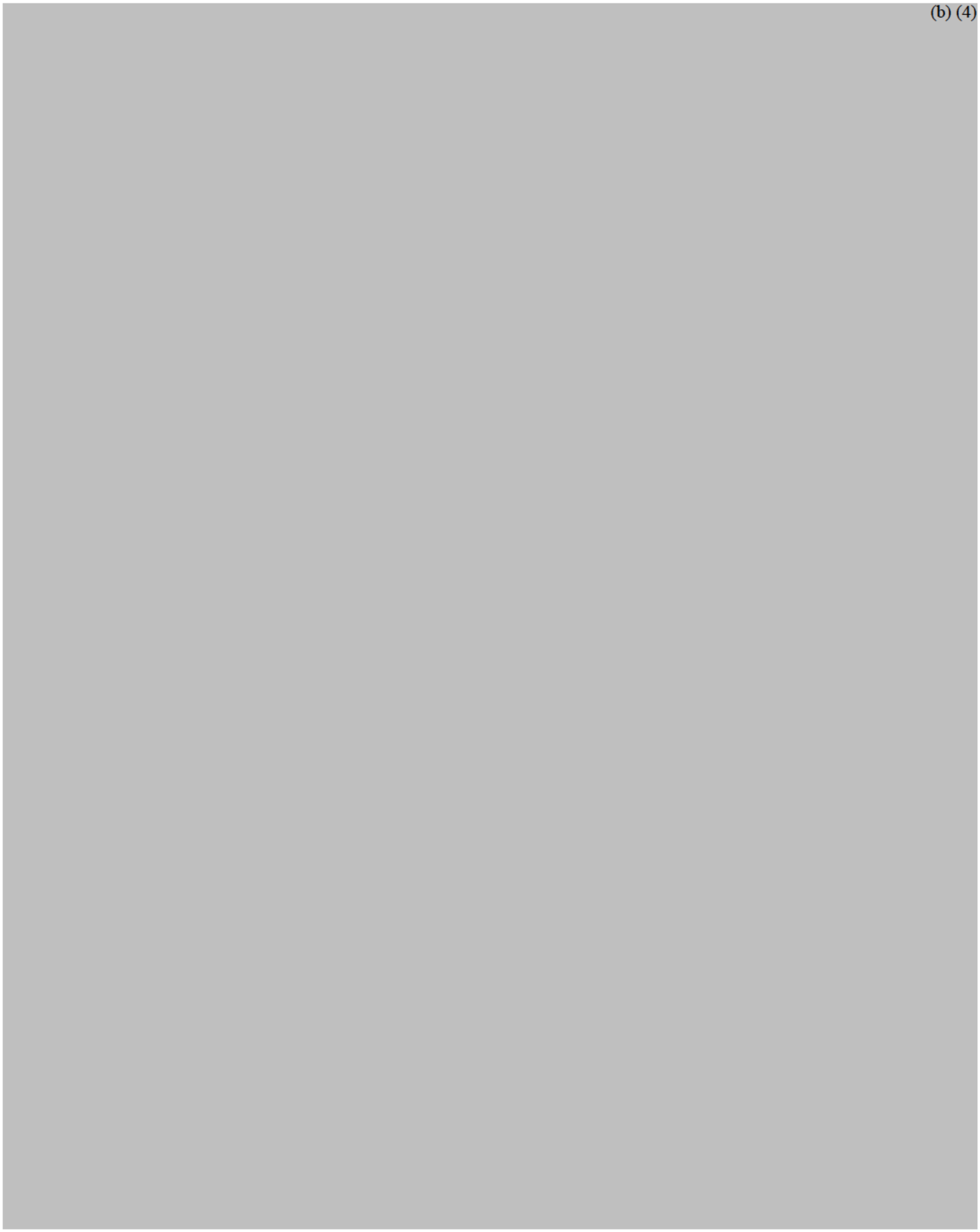

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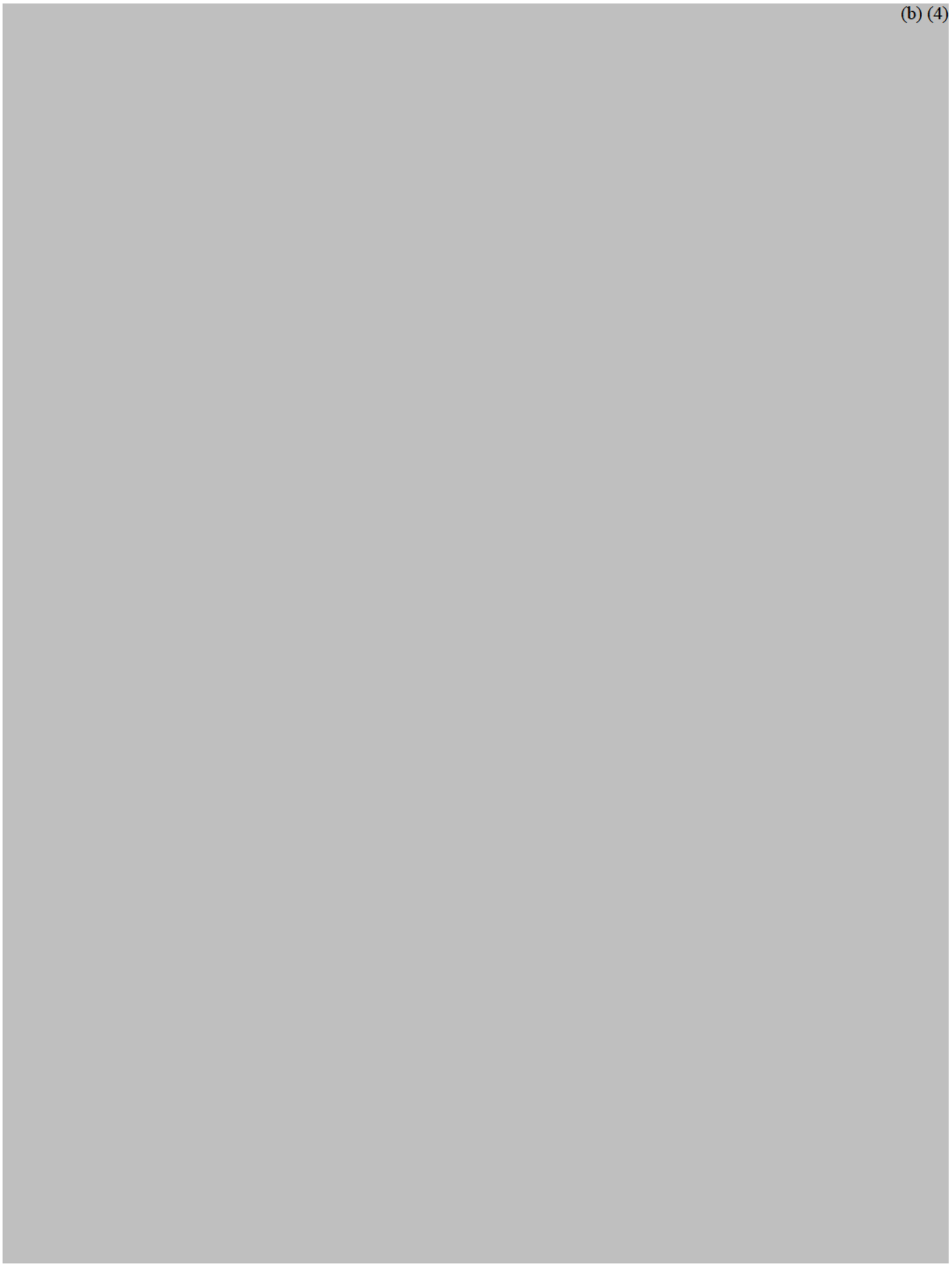

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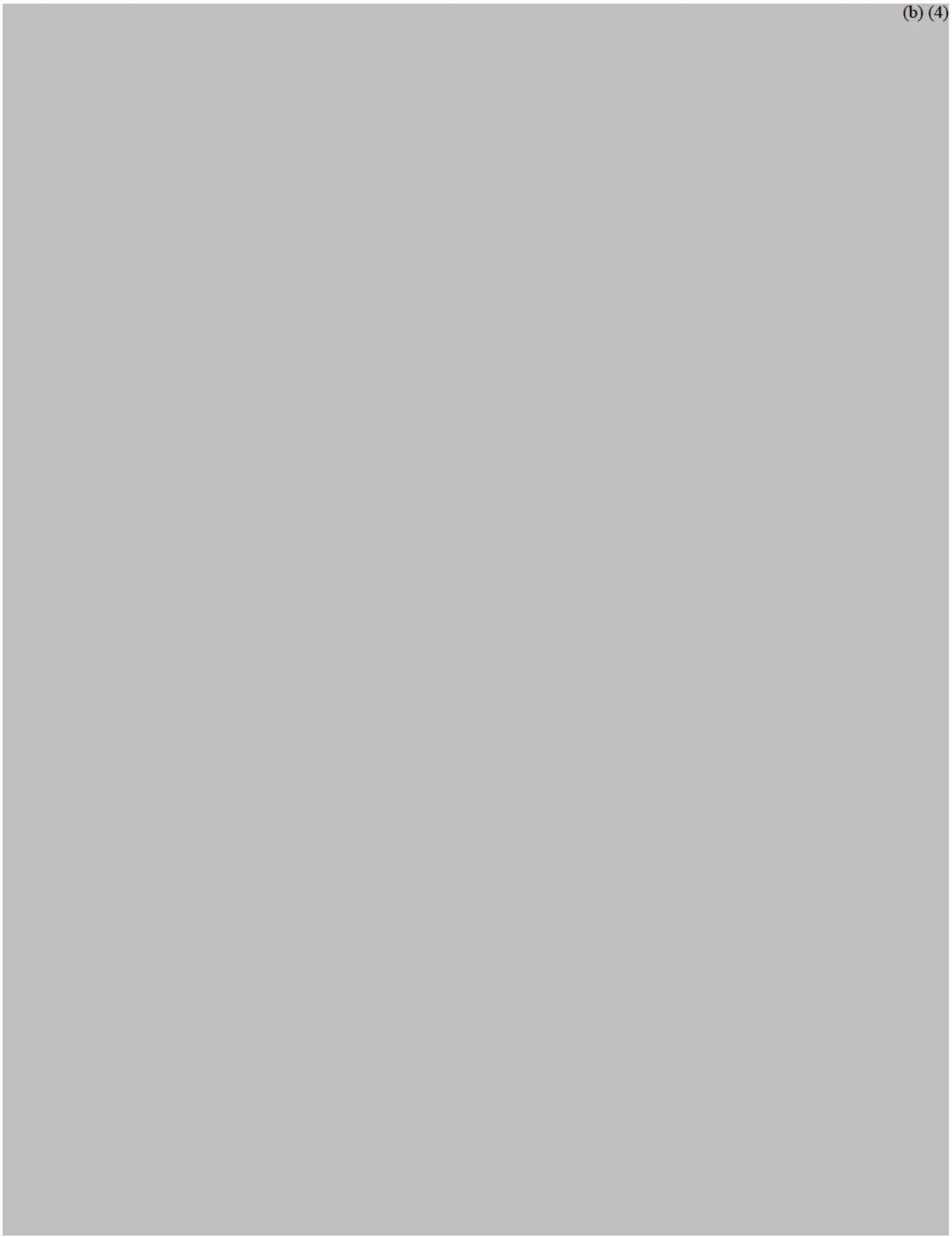

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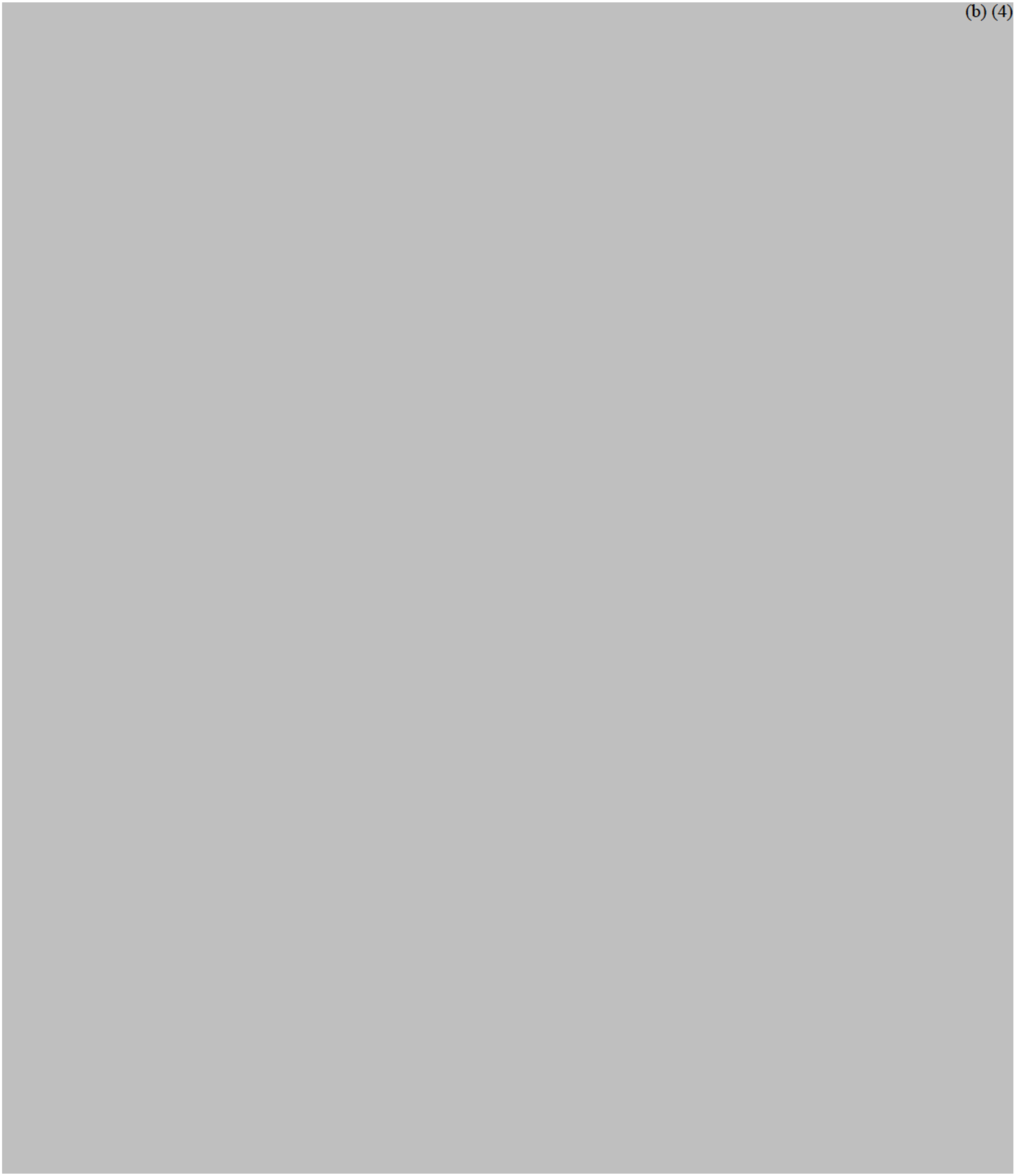

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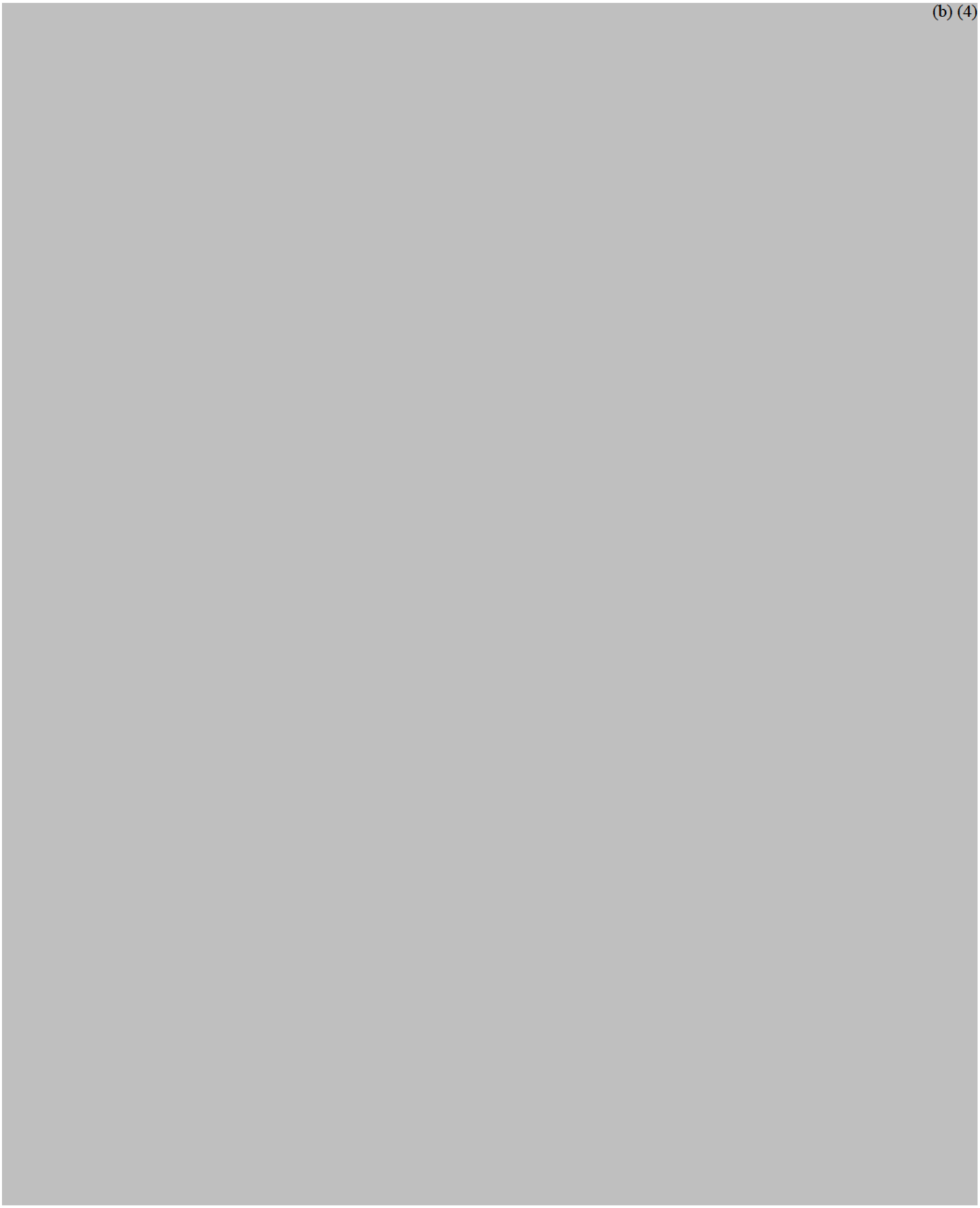

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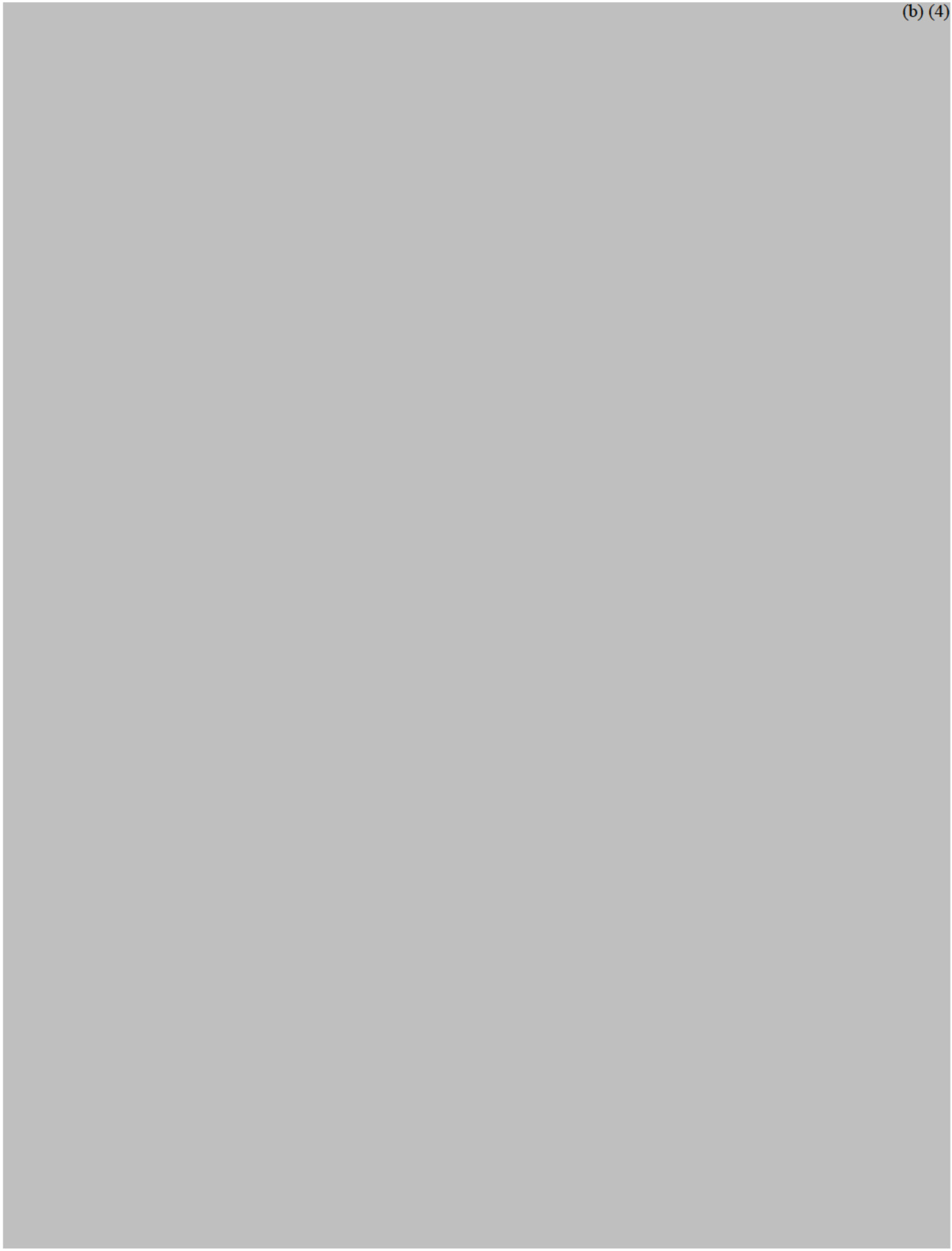

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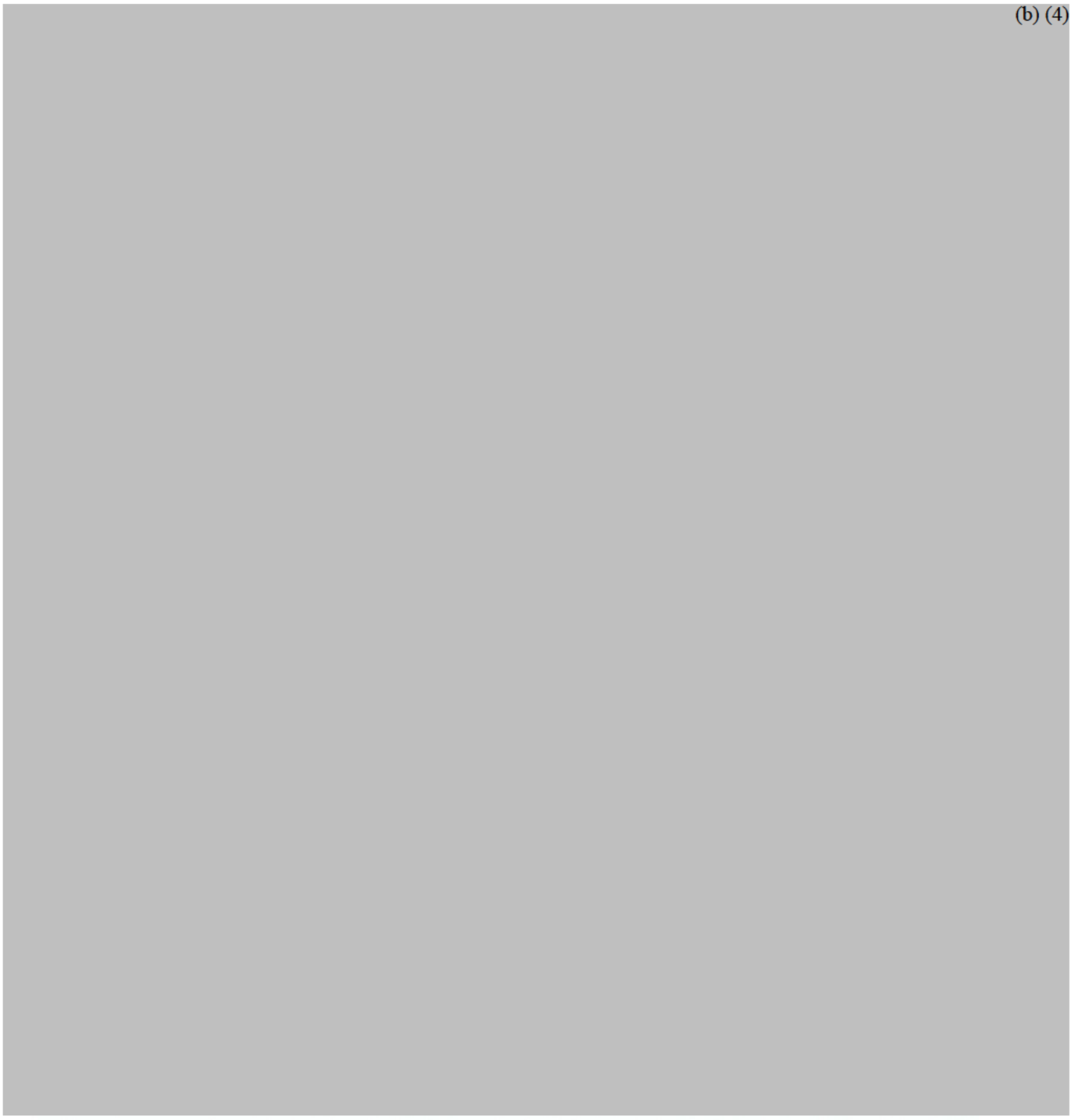
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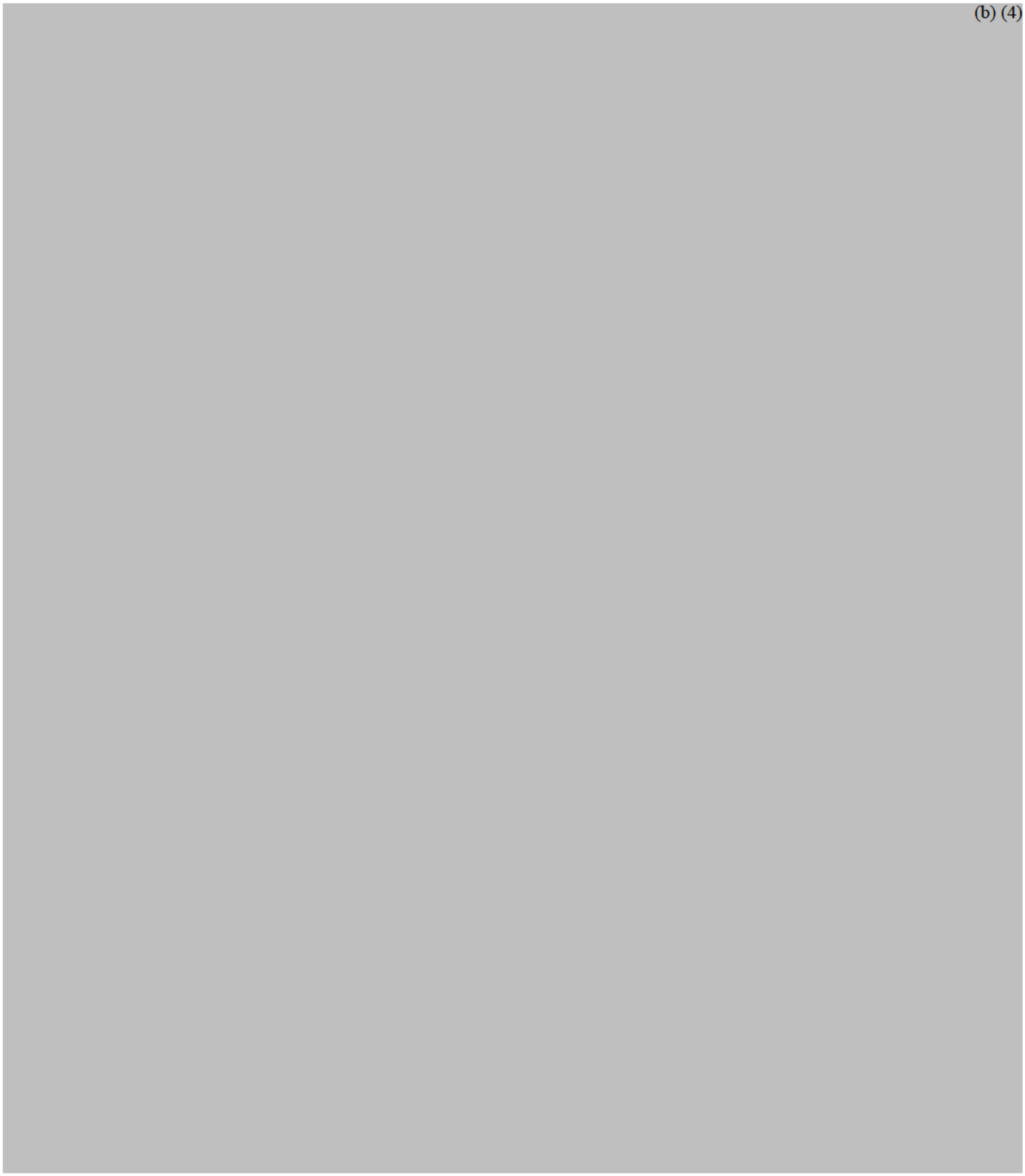
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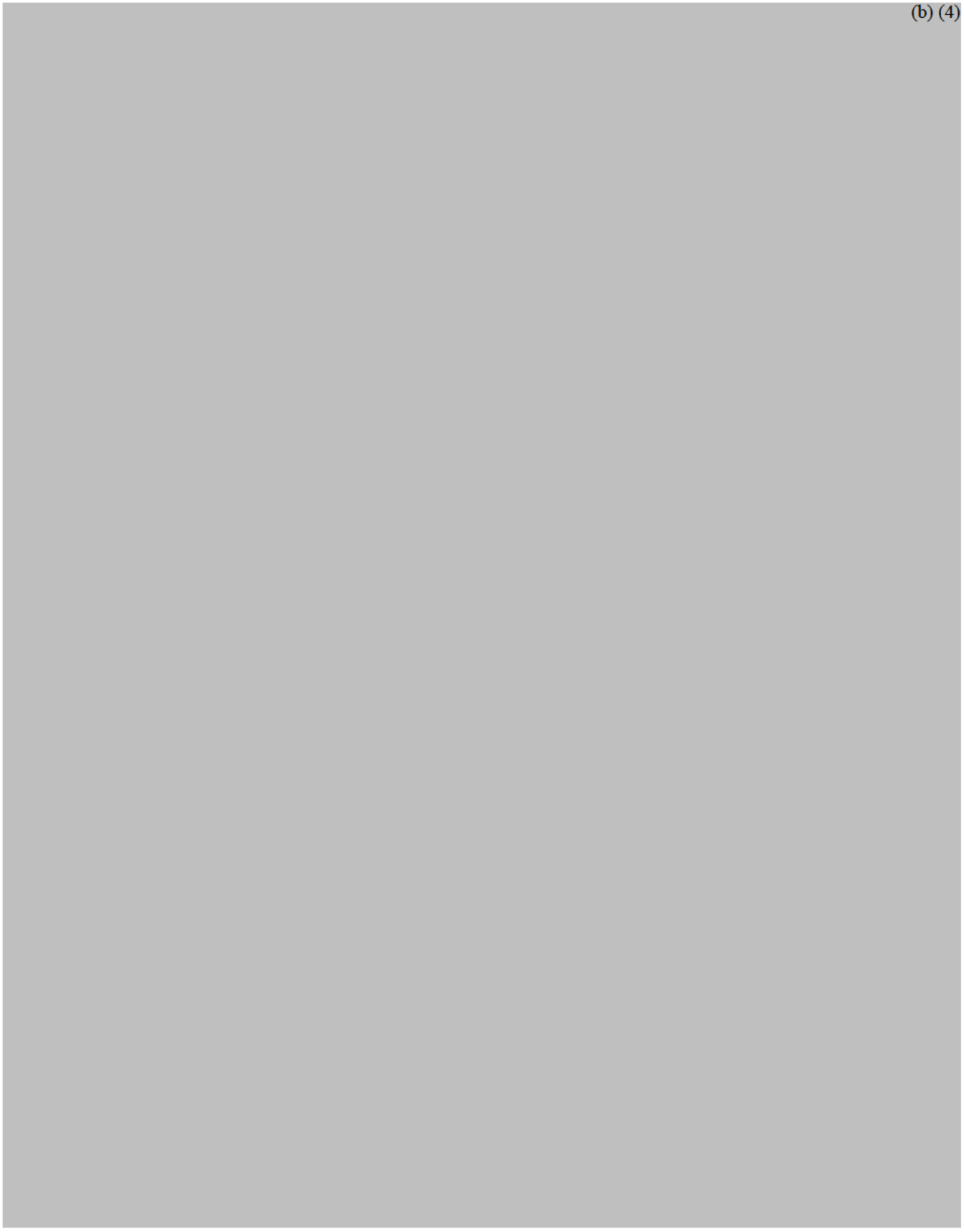
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## APPENDIX 2 Full Descriptions of the FERMENTATION and Manufacturing Process (CONFIDENTIAL)

### A. Manufacturing Process

L-methionine (L-Met) is manufactured by a two-step fermentation process. (b) (4)

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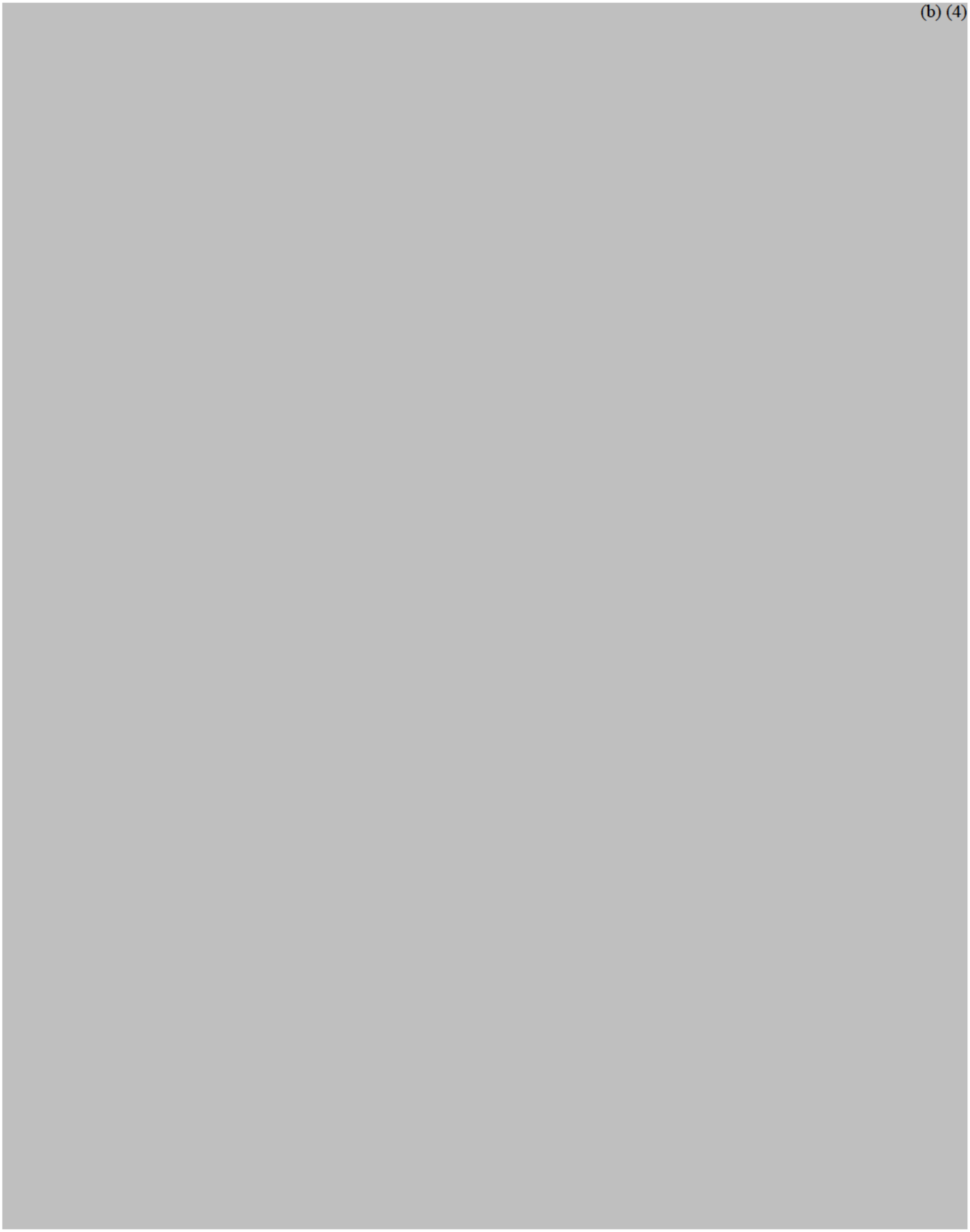

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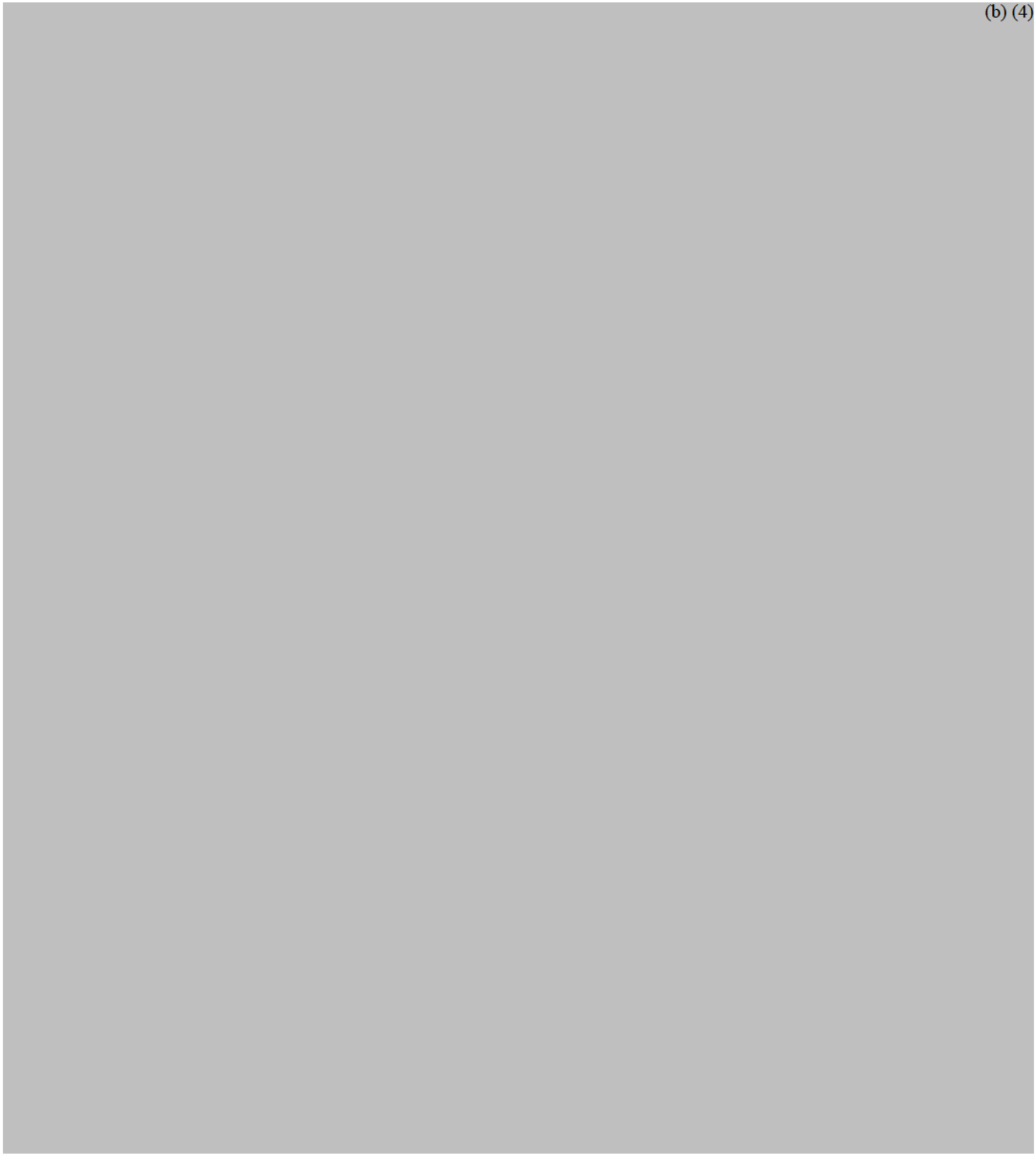


## B. Infor mation of Raw material

**APPENDIX 3 Acute Toxicity Report (CONFIDENTIAL)**

# REPORT

# L-Methionine (90%):

# Acute Oral Toxicity in the Rat - Fixed Dose Method

**Study Director:** (b) (4)

**Test Facility:** (b) (4)

**Sponsor:** **CJ CheilJedang Corporatio**
BIO R&D Institute
92, Ga-yang Dong
Kang-seo Gu
Seoul 157-724
SOUTH KOREA

**Study Number:** **41501198**

**Study Completion Date:** ☐

## STUDY DIRECTOR STATEMENT OF GLP COMPLIANCE

(b) (4)

Study Number : 41501198
Study Title : L-Methionine (90%):
Acute Oral Toxicity in the Rat - Fixed Dose Method

With the exception noted below this study was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These principles are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI).

No analysis was carried out to determine the homogeneity, concentration or stability of the test item formulation. The test item was formulated within two hours of it being applied to the test system; it is assumed that the formulation was stable for this duration. This exception is considered not to affect the purpose or integrity of the study.

This report fully and accurately reflects the procedures used and data generated. There were no circumstances considered to have affected the integrity of the study or the validity of the data.

Study Director: (b) (4)

.....

Date:

# QUALITY ASSURANCE STATEMENT

Study Number : 41501198
Study Title : L-Methionine (90%):
Acute Oral Toxicity in the Rat - Fixed Dose Method

The general facilities and activities are inspected at least once a year and the results are reported to the relevant responsible person and management.

Study-related procedures conducted at the test facility were audited and inspected. The details of these audits and inspections are given below.

| **Dates and Types of QA Inspections** | | | **Reported to the relevant Study Director and Test Facility Management** |
|---|---|---|---|
| Date of Inspection | Type of Inspection | Phase Inspected | Report Date |
| 13 July 2015 | Study Plan Verification | N/A | 13 July 2015 |
| 04 August 2015 | Process – based | Test Item Preparation | 04 August 2015 |
| 11 August 2015 | Process – based | Test System Preparation and Application | 11 August 2015 |
| 05 August 2015 | Process – based | Assessment of Response | 05 August 2015 |
| 05 August 2015 | Process – based | Necropsy | 05 August 2015 |
| 26 October 2015 | Report Audit | N/A | 26 October 2015 |

This statement confirms that this report reflects the raw data and the procedures followed.
Quality Assurance:

.....
Date:

# SUMMARY


## Introduction

The study was performed to assess the acute oral toxicity of the test item in the Wistar strain rat.

## Methods

Following a sighting test at a dose level of 2000 mg/kg, an additional four fasted female animals were given a single oral dose of test item, as a suspension in arachis oil BP, at a dose level of 2000 mg/kg body weight. Clinical signs and body weight development were monitored during the study. All animals were subjected to gross necropsy.

## Results

***Mortality.*** There were no deaths.

***Clinical Observations.*** There were no signs of systemic toxicity.

***Body Weight.*** All animals showed expected gains in body weight.

***Necropsy.*** No abnormalities were noted at necropsy.

## Conclusion

The acute oral median lethal dose (LD50) of the test item in the female Wistar strain rat was estimated to be greater than 2000 mg/kg body weight (Globally Harmonized Classif ication System Unclassified).

# GENERAL INFORMATION

**Schedule**
Experimental Starting Date: 11 August 2015
Experimental Completion Date: 03 September 2015

**Animal Welfare**
The study was designed and conducted to cause the minimum suffering or distress to the animals consistent with the scientific objectives and in accordance with the Envigo - Shardlow policy on animal welfare and the requirements of the United Kingdom's Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012. The conduct of the study may be reviewed, as part of the Envigo - Shardlow Ethical Review Process.
The study was conducted in accordance with the UK Home Office Guidance document o n Regulatory Toxicology and Safety Evaluation Studies and the OECD guidance docume nt on recognition, assessment and use of clinical signs as humane endpoints for experim ental animals used in safety evaluation.

**Deviations from Study Plan**
There were no deviations (unplanned changes) from the study plan.

**Archiving**
Records and documentation relating to this study (including electronic records) will be maintained in the archives of Envigo - Shardlow for a period of 2 years from the date on which the Study Director signs the final report. This will include but may not be limited to the study plan, raw data, electronic records and other samples and specimens generated during the course of this study.

At termination of the aforementioned period, records may be transferred to the Sponsor or the archiving period extended as mutually agreed. Further retention or return of the materials and data will be chargeable to the Sponsor. No data will be discarded without contacting the Sponsor to obtain their written consent.
A copy of the study plan and final report will be archived indefinitely at Envigo - Shardlow.

In case records are transferred from the Testing Facility, the Sponsor should ensure that the materials and records in support of regulatory studies are retained and maintained u nder conditions that guarantee their integrity and continued access according to archivi ng requirements of the principles of GLP. The Sponsor should also ensure that such mat erials and records are retained for as long as required by relevant authorities.

## 1. INTRODUCTION AND PURPOSE

The study was performed to assess the acute oral toxicity of the test item in the Wistar strain rat.

### 1.1 Guidelines / Regulations

This study was designed to be compatible with the procedures indicated by the following internationally accepted guidelines and recommendations:

- OECD Guideline for Testing of Chemicals No 420 "Acute Oral Toxicity - Fixed Dose Method" (2001)
- Method B1 bis Acute Toxicity (Oral) of Commission Regulation (EC) No. 440/2008

## 2. TEST ITEM

Information as provided by the Sponsor. A Certificate of Analysis supplied by the Sponsor is given in Appendix 3.

(b) (4)

## 3 MATERIALS AND METHODS

### 3.1 Test System

#### 3.1.1 Animals and Animal Husbandry

Female Wistar (RccHan™:WIST) strain rats were supplied by Envigo RMS (UK) Limited, Oxon, UK. On receipt the animals were randomly allocated to cages. The females were nulliparous and non-pregnant. After an acclimatization period of at least 5 days the animals were selected at random and given a number unique within the study by indelible ink-marking on the tail and a number written on a cage card. At the start of the study the animals were 8 to 12 weeks of age. The body weight variation did not exceed ±20% of the mean body weight at the start of treatment.

The animals were housed in groups of up to four in suspended solid-floor polypropylene cages furnished with woodflakes. With the exception of an overnight fast immediately before dosing and for approximately 3 to 4 hours after dosing, free access to mains drinking water and food (2014C Teklad Global Rodent diet supplied by Envigo RMS (UK) Limited, Oxon, UK) was allowed throughout the study. The diet, drinking water and bedding were routinely analyzed and were considered not to contain any contaminants that would reasonably be expected to affect the purpose or

integrity of the study.

The temperature and relative humidity were set to achieve limits of 19 to 25 °C and 30 to 70% respectively. The rate of air exchange was at least fifteen changes per hour and the lighting was controlled by a time switch to give 12 hours continuous light and 12 hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

### 3.1.2 Justification

Rats are the preferred species of choice as historically used for safety evaluation studies and are specified in the appropriate test guidelines.

## 3.2 Test Item Formulation and Experimental Preparation

For the purpose of the study the test item was freshly prepared, as required, as a suspension in arachis oil BP. Arachis oil BP was used because the test item did not dissolve/suspend in distilled water.

The test item was formulated within 2 hours of being applied to the test system. It is assumed that the formulation was stable for this duration.

No analysis was conducted to determine the homogeneity, concentration or stability of the test item formulation. This is an exception with regard to GLP and has been reflected in the GLP compliance statement.

## 3.3 Procedure

Using available information on the toxicity of the test item, 2000 mg/kg was chosen as the starting dose.

A single animal was treated as follows:

| Dose Level (mg/kg) | Concentration (mg/mL) | Dose Volume (mL/kg) | Number of Rats |
|---|---|---|---|
| | | | Female |
| 2000 | 200 | 10 | 1 |

In the absence of mortality at a dose level of 2000 mg/kg, an additional group of animals was treated as follows:

| Dose Level (mg/kg) | Concentration (mg/mL) | Dose Volume (mL/kg) | Number of Rats |
|---|---|---|---|
| | | | Female |
| 2000 | 200 | 10 | 4 |

A total of five animals were therefore treated at a dose level of 2000 mg/kg in the study.

All animals were dosed once only by gavage, using a metal cannula attached to a graduated syringe. The volume administered to each animal was calculated according to the fasted body weight at the time of dosing. Treatment of animals was sequential. Sufficient time was allowed between each dose group to confirm the survival of the previously dosed animals.

Clinical observations were made 30 minutes, 1, 2, and 4 hours after dosing and then daily for 14 days. Morbidity and mortality checks were made twice daily.

Individual body weights were recorded on Day 0 (the day of dosing) and on Days 7 and 14.

At the end of the observation period the animals were killed by cervical dislocation. All animals were subjected to gross necropsy. This consisted of an external examination a nd opening of the abdominal and thoracic cavities. The appearance of any macroscopi c abnormalities was recorded. No tissues were retained.

### 3.4 Evaluation of Data

The test item was classified according to Annex 3 of the OECD Guidelines for Testing of Chemicals No. 420 "Acute Oral Toxicity - Fixed Dose Method" (adopted 17 December 2001) as shown in the Flow Chart in Appendix 2.

Evaluation of data included identification of the number of animals that died during the study (or that were killed for humane reasons), and determination of the nature, severity, onset and duration of the toxic effects. If possible, the signs of evident toxicity were described. Evident toxicity refers to the toxic effects of sufficient severity that administration of the next higher dose level could result in development of severe signs of toxicity and probable mortality. Effects on body weights and abnormalities noted at necropsy were also identified.

Using the mortality data obtained, an estimate of the acute oral median lethal dose (LD50) of the test item was made.

## 4 RESULTS

Individual clinical observations and mortality data are given in Table 1.

### 4.1 Mortality

There were no deaths.

### 4.2 Clinical Observations

No signs of systemic toxicity were noted during the observation period.

### 4.3 Body Weight

Individual body weights and body weight changes are given in Table 2.
All animals showed expected gains in body weight over the observation period.

### 4.4 Necropsy

Individual necropsy findings are given in Table 3.
No abnormalities were noted at necropsy.

## 5 CONCLUSIONS

The acute oral median lethal dose (LD50) of the test item in the female Wistar strain rat was estimated to be greater than 2000 mg/kg body weight (Globally Harmonized Classification System ☐ Unclassified).

## 6 REFERENCES

ENVIRONMENT DIRECTORATE, ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT OECD (2000) *No. 19 Guidance Document on the Recognition, Assessment and use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation*. Paris: OECD Environmental Health and Safety Publications Series on Testing and Assessment.

The Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012.

UK HOME OFFICE (2005) Guidance on the Conduct of Regulatory Toxicology and Safety Evaluation Studies.

# TABLE

Table 1 Individual Clinical Observation and Mortality Data

| Dose Level mg/kg | Animal Number and Sex | Effects Noted After Dosing (Hours) | | | | Effects Noted During Period After Dosing (Days) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ½ | 1 | 2 | 4 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | |
| 2000 | 1-0 Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2-0 Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2-1 Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2-2 Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 2-3 Female | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 2 Individual Body Weights and Body Weight Changes

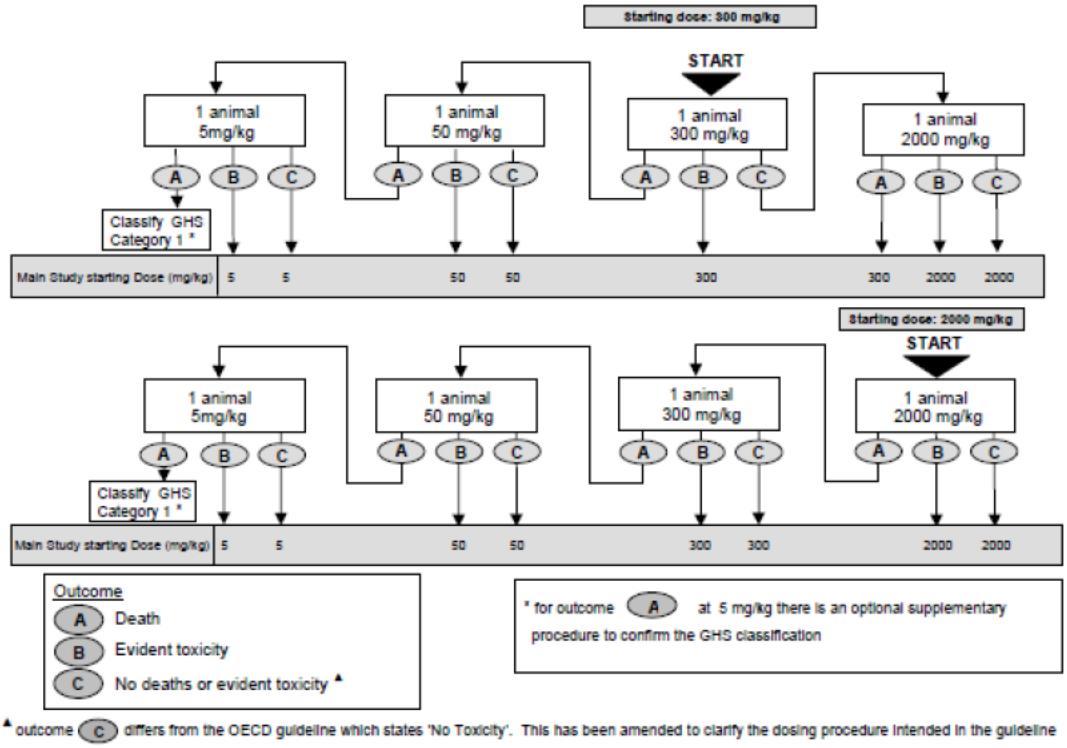
| Dose Level mg/kg | Animal Number and Sex | Body Weight (g) at Day | | | Body Weight Gain (g) During Week | |
|---|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 1 | 2 |
| 2000 | 1-0 Female | 188 | 204 | 209 | 16 | 5 |
| | 2-0 Female | 165 | 182 | 197 | 17 | 15 |
| | 2-1 Female | 162 | 176 | 189 | 14 | 13 |
| | 2-2 Female | 160 | 175 | 189 | 15 | 14 |
| | 2-3 Female | 160 | 174 | 191 | 14 | 17 |

Table 3 Individual Necropsy Findings

| Dose Level mg/kg | Animal Number and Sex | Time of Death | Macroscopic Observations |
|---|---|---|---|
| 2000 | 1-0 Female | Killed Day 14 | No abnormalities detected |
| | 2-0 Female | Killed Day 14 | No abnormalities detected |
| | 2-1 Female | Killed Day 14 | No abnormalities detected |
| | 2-2 Female | Killed Day 14 | No abnormalities detected |
| | 2-3 Female | Killed Day 14 | No abnormalities detected |

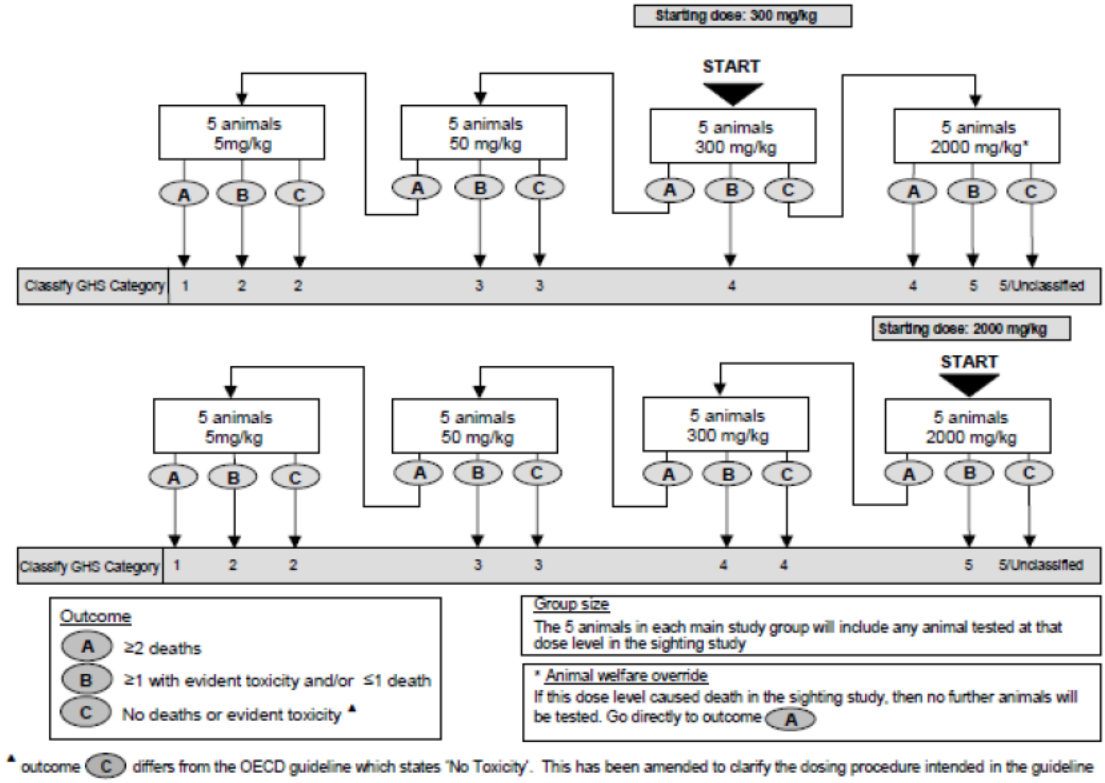
# APPENDICES

## Appendix A. Flow Chart for the Sighting Test




▲ outcome C differs from the OECD guideline which states 'No Toxicity'. This has been amended to clarify the dosing procedure intended in the guideline

## Appendix B. Flow Chart for the Main Test



Appendix C. Certificate of Analysis

CJ Research Institute of Biotechnology
92-1, Gayang-dong, Gangseo-gu, Seoul, 157-724, Korea
www.cj.co.kr
TEL : 02) 3660-0733 FAX : 02) 3660-0501

CJ CHEILJEDANG BIO

## Certificate of analysis

| | | | |
|---|---|---|---|
| Certificate No. | 2015-AN-016 | Receipt No. | 2015-AR-016 |
| Client | (b) (4) | Date of Receipt | 2015-03-23 |
| Client Name | | Date of Test | 2015-03-25 |
| Client Tel | +60(3)7832-0687 | Use of Report | Reference test |
| Client Address | Oasis Office Unit Nr A-05-09, 2 Jalan Pju 1a/7a, Ara Damansara, 47301 Petaling Jaya,Selangor Darul Ehsan, Malaysia. | | |

| | |
|---|---|
| Test Sample | (b) (4) |
| Manuf. Date | |
| Expiry Date | |
| Lot. No | |
| Quantity (kg) | |
| Test Item(s) | |
| L-Methionine(Dry Matter) | |

(b) (4)

| | | |
|---|---|---|
| | | |
| | | |

* Temperature : (22~28) ℃, Relative Humidity : (30~50) %
* N.D : not detected (not quantifiable)
* The results shown in this test report refer only to the sample tested unless otherwise stated.

The Test (b) (4) ept in full.

Tested by

Approved by Technical Manager (b) (4)

Jun, 30, 2015

CJ Research Institute of Biotechnology, BIO)Analysis Team

CJ BIO-AD form 100-01 REV 01

Appendix D. Monitoring Authority Statement of GLP Compliance

DH Department of Health

**THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM**

**GOOD LABORATORY PRACTICE**

**STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 2004/9/EC**

(b) (4)

(b) (4)

**DATE OF INSPECTION**

An inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above test facility as part of the UK Good Laboratory Practice Compliance Monitoring Programme.

This statement confirms that, on the date of issue, the UK Good Laboratory Practice Monitoring Authority were satisfied that the above test facility was operating in compliance with the OECD Principles of Good Laboratory Practice.

This statement constitutes a Good Laboratory Practice Instrument (as defined in the UK Good Laboratory Practice Regulations 1999).

24/9/15

**Dr. Andrew J. Gray**
**Head, UK GLP Monitoring Authority**

MHRA

T-4


# Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
Woodbridge, VA 22192-5755
703 590 7337 (Fax 703 580 8637)
CFR@cfr-services.com

Since 1973
*consultants to the regulated industry*


January 19, 2018

Geoffrey Wong
Leader, Ingredient Safety Team
Division of Animal Feeds (HFV-224)
Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

Subject: Animal GRAS Notification
L-Methionine 90% (GRN 24)
AMENDMENT

Notifier: CheilJedang Corporation

Dear Mr. Wong,

We are amending the information provided to the agency specific t the GRAS conclusion as made by CheilJedang Corporation L-methionine 90% produced by a bioengineered Escherichia coli K-12 as a source of Methionine in animal diets on June 28, 2017 (GRN 24). This amendment is made based on a telephone conference that included Geoff Wong, Thomas Hendrick, and Rial Christensen of the Division of Animal Feeds .

The initial topic was specific to the genetic engineering data to support the modification of the Escherichia coli K-12 specific to modifications required for the fermentation process resulting in the highly purified methionine ingredient. Dr. Christensen stated that unless additional information was provided, the final review letter as issued to CheilJedang Corporation would include some of the basic purification steps (he indicated that this was consistent with the approach taken under GRN 16). CheilJedang Corporation is agreeing with the approach that would necessitate the general description of the purification steps for the post-fermentation product that results in the highly purified methionine product to be included in the FDA response to the notice.

1. **Dr. Hendricks requested that the CheilJedang Corporation the Analytical data to support the specifications as provided in table 3 (page 7).** This data is provided in Annex 1..

SUMMARY TABLE OF BATCH ANALYSIS

| Test | Units | (b) (4) |
|---|---|---|
| Methionine | % | |
| Moisture | % | |
| Ash | % | |
| Ammonium | % | |
| Free amino acids | % | |
| Organic acids | % | |
| Ions | % | |
| Total of quantified components[1] | % | |

[1]: by calculation (%)

Please refer to the attached the analytical report (Annex 1).

2. **Dr. Hendricks requested that a complete list of raw materials that are used in the manufacture (fermentation) of the product.**

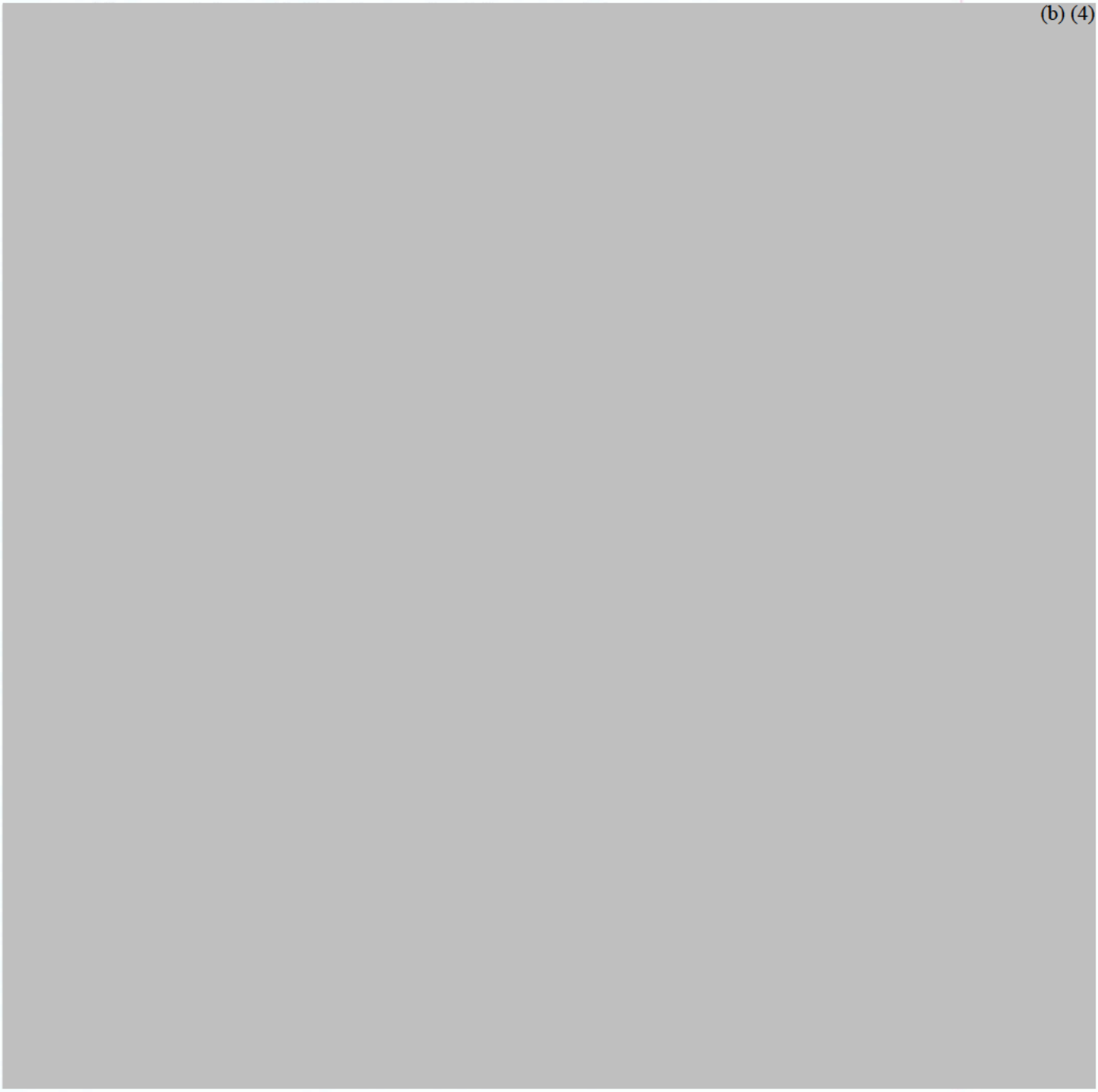
(b) (4)

January 19, 2108

(b) (4)

January 19, 2108

(b) (4)



3. **Dr. Hendricks requested identification of the antifoam used in the fermentation including the specific chemical compound.**

(b) (4)

This antifoam ingredient has been reviewed by the Division of Animal Feed and was found to be suitable for the manufacture of animal feed ingredients (see FDA letter to Gary Yingling, ETA Association, September 11, 2003).

**4. The Division requested a signed statement that says that all substances used in the manufacture and formulation of the GRAS substance (L-methionine 90% produced by a bioengineered Escherichia coli K-12) are suitable for animal feed or CJ has determined they are GRAS for the intended use.**
Please refer to the attached file. (Annex 4)

We believe that this information responds to all the concerns addressed in our teleconference and we are looking forward to your final response. Please contact us immediately shoud you have any further questions on the notice.

Sincerely

Kristi O. Smedley, Ph.D.
Consultant to CheilJedang Corporation

**REFERENCES**

FDA SCOGS (Select Committee on GRAS Substances)
https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=scogs

EFSA Feed Material Catalogue http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0068&from=EN

CFIA Update of Permitted Feeds (January, 2018)

**ATTACHMENTS:**

Annex 1 Batch analysis data
Annex 2 Certificate of Analysis (b) (4)
Annex 3 MSDS (b) (4)
Annex 4 Signed statement on ingredients
Annex 5 Analytical method of Validation of Methyl Mercaptan in L-methionine

**Doody, Carissa** T-5

**From:** Hendricks, Thomas T
**Sent:** Monday, March 19, 2018 11:49 AM
**To:** Doody, Carissa
**Cc:** Wong, Geoffrey K
**Subject:** FW: AGRN 240--L-methionine 90%
**Attachments:** AOAC 999.13.pdf

**Follow Up Flag:** Follow up
**Flag Status:** Flagged

Hi Carissa,

Here is an amendment to M055, please file.

Thank you
Tom

M. Thomas Hendricks, PhD, MPH
CAPT, USPHS
Regulatory Review Officer
Division of Animal Feeds
FDA/CVM/OS&C
7519 Standish Place, HFV-224
Rockville, Maryland 20855
email: thomas.hendricks@fda.hhs.gov
(240) 402-5925 (voicemail) (new telephone number)
(240) 453-6882 (fax)



---

**From:** Kristi Smedley [mailto:smedley@cfr-services.com]
**Sent:** Monday, March 19, 2018 11:35 AM
**To:** Wong, Geoffrey K <Geoffrey.Wong@fda.hhs.gov>; Hendricks, Thomas T <Thomas.Hendricks@fda.hhs.gov>
**Cc:** Keith D. Haydon <keith.haydon@cj.net>
**Subject:** AGRN 240--L-methionine 90%

Dr. Hendricks and Mr. Wong:

In response to your phone call on Thursday, March 15, 2017, I have determined that the analytical method CJ uses to confirm methionine concentration in the final product (including the values as provided in the notice of the GRAS conclusion) is AOAC 999.13.

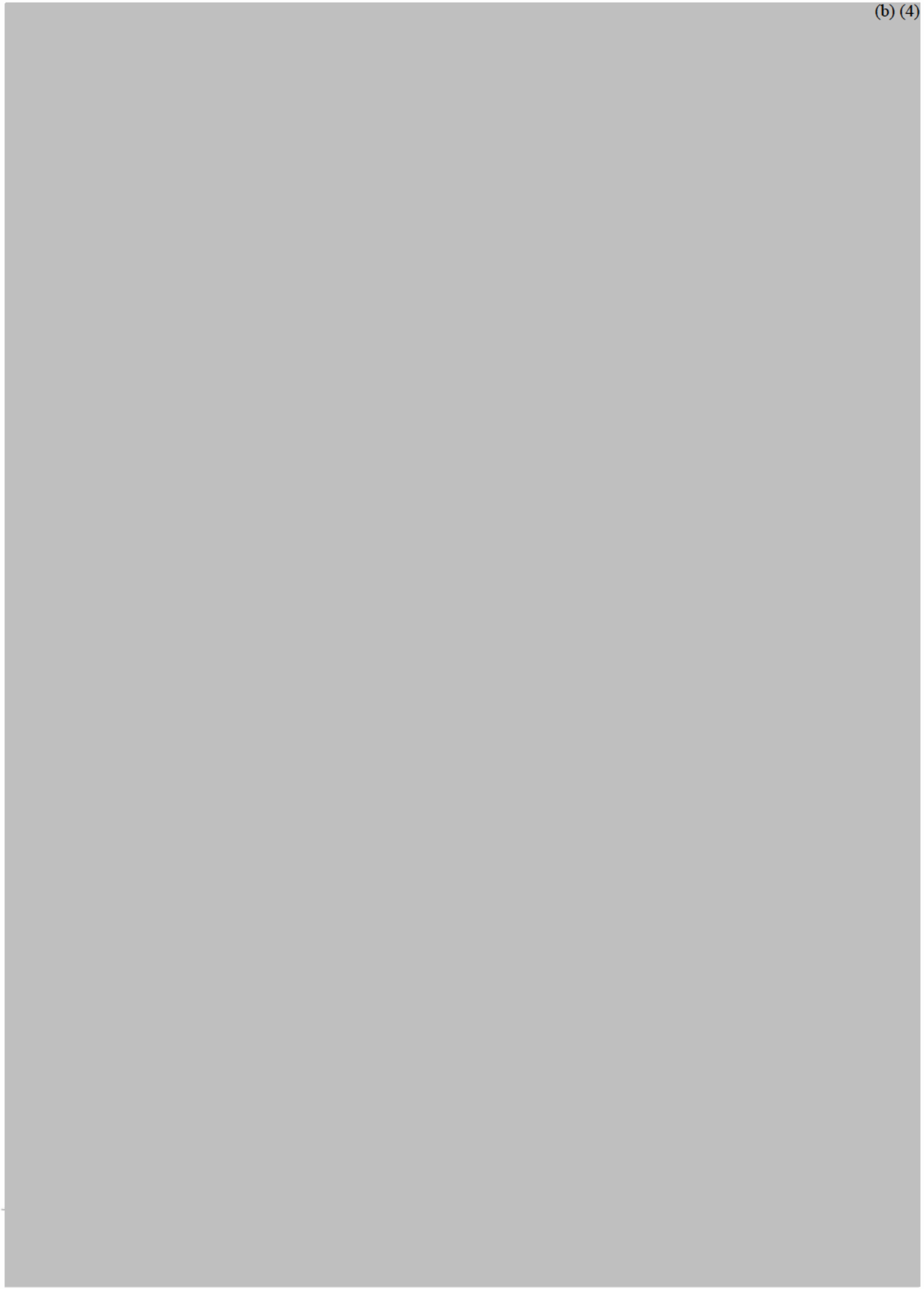
For you convenience I have provided a copy of the method.

Please let us know if you have any additional clarification or other concerns on the submission.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell 703-786-7674
Fax 703-580-8637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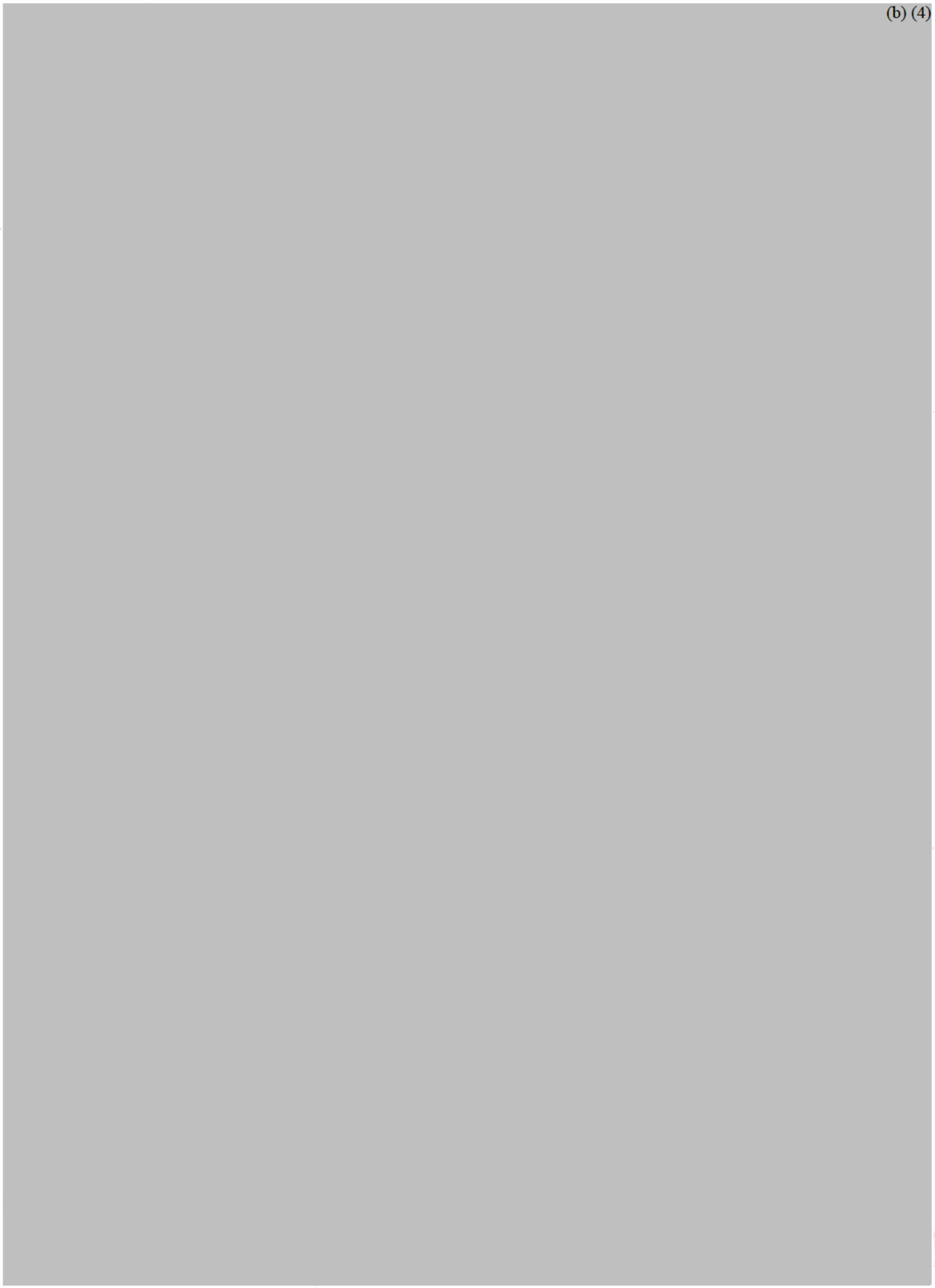
(b) (4)

[redacted]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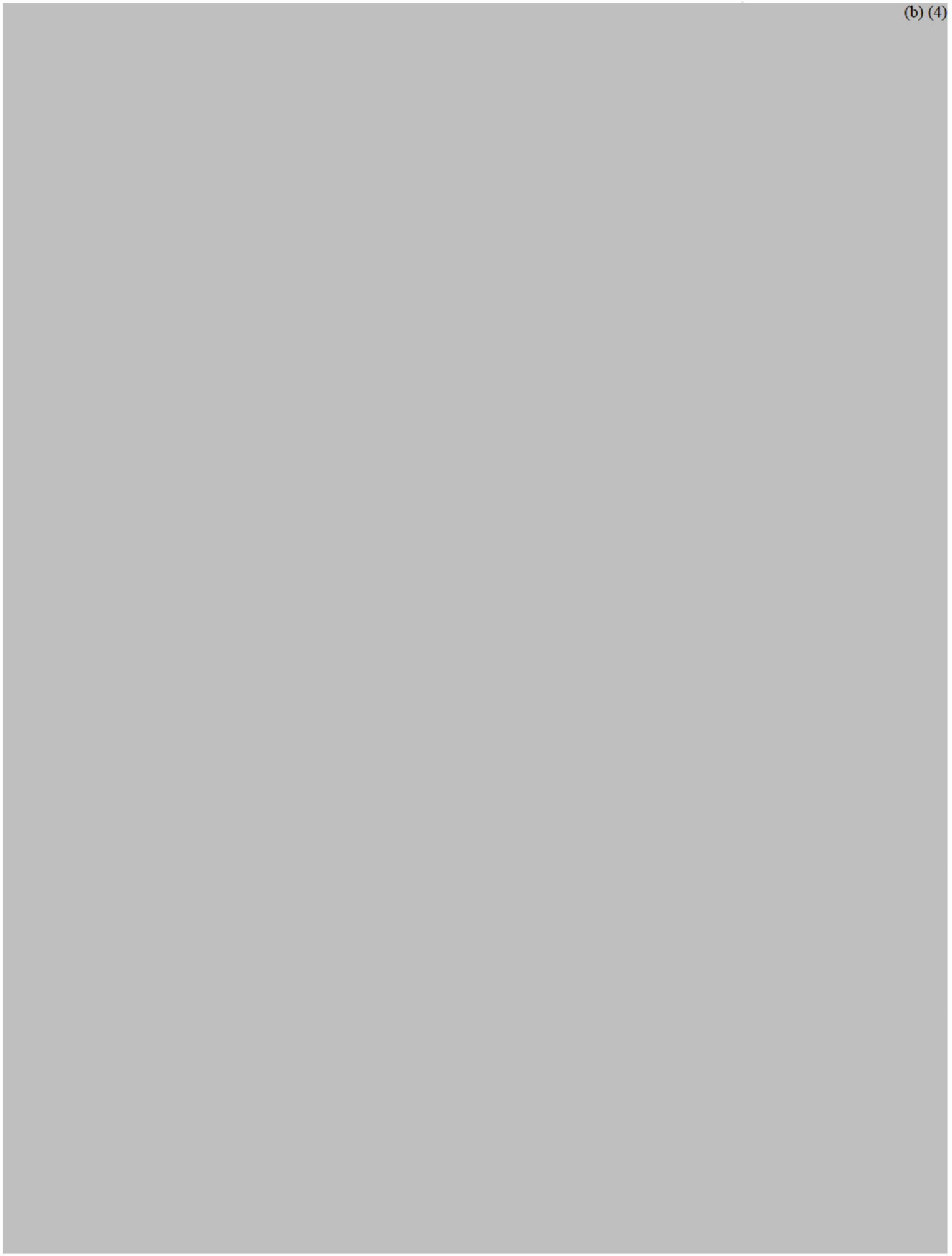
(b) (4)

(b) (4)